ENDOCRINOLOGY

VOLUME 68

JANUARY-JUNE, 1961

ENDOCRINOLOGY

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PUBLISHED FOR
THE ENDOCRINE SOCIETY

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George Banta Company, Inc.
Menasha, Wisconsin

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ENDOCRINOLOGY

VOLUME 68 JANUARY, 1961 NUMBER 1

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A SIMPLE METHOD FOR THE PURIFICATION OF SHEEP ACTH[1]

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ABSTRACT

A simple method is described for the preparation from sheep pituitaries of ACTH of 100 U.S.P. units/mg. with a yield of 50 mg./kg. wet weight of glands. The method lnvolves aqueous extraction of ground sheep pituitaries with 0.1N HCl, removal of coagulated proteins at 85° C and pH 5.6, oxycellulose adsorption of the hormone from the supernatant and elution with 0.1N HCl, followed by column chromatography on IRC-50. The oxycellulose eluate and the IRC-50 column eluate are both dialyzed. The ACTH is characterized in hypophysectomized rats by its ability to increase the adrenal weights and repair the morphology, decrease the thymus weights, deplete adrenal ascorbic acid, and stimulate calorigenesis and erythropoiesis.

THE following method for the preparation of highly active adrenocorticotrophic hormone (ACTH) from whole sheep pituitaries (100 U.S.P. units/mg.) grew out of the study in this laboratory of erythropoietically active principles of the pituitary (1). While most of the procedures reported here have been used previously by others, the method is proposed because of its simplicity and the good yield and potency of the product.

MATERIALS AND METHODS

Four kilograms of twice ground frozen whole sheep pituitaries were extracted in two portions of two kilograms each with 4 liters of 0.1N HCl for 1 hr. in the cold. The suspension (pH 3.8) was immediately adjusted to pH 5.6 with 6N NaOH and heated over an open flame, with stirring, to 85° C in order to coagulate the major portion of the protein. The clear supernatant separated by filtration with suction was immediately cooled to room temperature. The residue was re-extracted for 10 minutes with 4 l. of H_2O at room temperature. The combined filtrates from both lots measured about 16 l. Glacial acetic acid was added to the filtrates at room temperature to give a final concentration of 0.1N acetic acid and 10 gm. of oxycellulose was added. After 20 hr. of stirring at room

Received March 12, 1960.

[1] The work here reported was supported in part by a grant from the United States Public Health Service, A-661.

temperature the oxycellulose was allowed to settle, the supernatant was decanted, and the oxycellulose was collected by centrifugation. The oxycellulose was Eastman Oxidized Cellulose Powder 11.97% COOH which had been washed prior to use with 0.1N HCl, then water and finally 1N acetic acid.

ACTH was eluted from the oxycellulose by stirring with 250 ml. of 0.1N HCl for 1 hr. and then successively with three 100 ml. portions of 0.1N HCl. The eluates were collected by filtration with suction, combined, adjusted to pH 7 with 6N NaOH and lyophilized. The dry material thus obtained was dissolved in approximately 100 ml. H_2O and dialyzed (with external stirring) against 12 l. portions of cold H_2O. The dialysis water was changed every 12 hr. during 48 hr. The dialyzed solution, including some precipitate formed during the dialysis, was then lyophilized.

The entire product was extracted in one lot with 300 ml. 0.05N $NaHCO_3$ and a small amount of insoluble material was removed by centrifugation. The clear extract was passed through a column of IRC-50 (2.2×30 cm.) which had previously been equilibrated with 0.05N $NaHCO_3$. The column chromatography was done in a cold room at 5° C. The column was then washed with 0.05N $NaHCO_3$ until the optical density at 278 mμ had decreased to 0.050 for a light path of 1 cm. (approximately 1 l. required). The ACTH was eluted with 0.15N $NaHCO_3$ until the optical density of the eluate had fallen to 0.100 for a 1 cm. light path (approximately 1 l. required). (In the initial experiments the ACTH was eluted with a concentration gradient of $NaHCO_3$ (0.05N to 0.15N) and was found to be distributed in two emerging peaks. Bioassay showed that the material contained in these two peaks were of equal adrenocorticotropic potency, and in subsequent experiments the column eluate was handled as one fraction.) The eluate was adjusted to approximately pH 6 with HCl and then dried by lyophilization. The dried eluate was taken up in 50 ml. H_2O and dialyzed with stirring in the cold for 32 hr. against 12 l. of H_2O with 4 changes. The dialyzed eluate was then dried by lyophilization.

The ACTH was assayed by its ability to increase adrenal weights of hypophysectomized rats. Long-Evans rats were hypophysectomized at 40 days of age and were maintained on a modified McCollum dry diet I (1). A supplemental wet mash of the diet was given once a day. They were injected intraperitoneally once a day for four days, beginning four days post-operatively, with 0.1 ml. of the hormone suspended in a 5% beeswax-peanut oil mixture to conform to the conditions of the test as described by Li (2). The animals were autopsied 24 hr. after the last injection. Completeness of hypophysectomy was determined at autopsy by examination of the pituitary site under a binocular dissecting microscope. The adrenal and thymus glands were weighed and the former were fixed for histological examination of frozen sections after staining with Sudan black. The adrenocorticotropic potency of the final product was also determined by the adrenal ascorbic acid depletion assay of Sayers *et al.* (3) and compared with a U.S.P. standard.

As this laboratory has been engaged in the analysis of the calorigenic (4, 5) and erythropoietic (1, 5) activity of ACTH, the column eluate ACTH was characterized with respect to these properties. Li has also used these methods to characterize α-corticotropin (2). Female rats of the Long-Evans strain were hypophysectomized at 28 days of age and were used for assay 45 days later in order to allow for the development of the post-hypophysectomy anemia. The hormone was injected subcutaneously in 0.1 ml. of 5% beeswax-peanut oil suspension once daily for fourteen days. Metabolic rates were measured terminally with a closed circuit respiration apparatus under standardized conditions (6). The blood volumes of the rats were determined at autopsy, 24 hours after the last injection of hormone by the Fe^{59} tagged red cell dilution method (7). Hematocrits were determined simultaneously and the red blood cell volume calculated by multiplying blood volume times hematocrit.

RESULTS

The yield of the oxycellulose eluate was 2.5 gm. (from 4 kg. wet weight of glands) with a potency of 9 U.S.P. units per mg. as determined by the adrenal weight assay. The yield of the final product obtained by column chromatography of the oxycellulose eluate on IRC-50 was 202 mg. or 50 mg. per kg. wet weight of glands. The potency determined by the adrenal weight assay was 100 U.S.P. units per mg. and by the ascorbic acid depletion assay was 97 U.S.P. units per mg. The yield of final product represents almost quantitative recovery of the activity present at the oxycellulose eluate stage, and compares advantageously with the yield of Li *et al.* (19) of 25 mg. per kg. wet weight of glands at the IRC-50 column stage. No attempt was made to determine quantitatively the activity present in the original crude acid extract of the glands. It was possible to recover additional ACTH activity from the crude extract by repeating the oxycellulose adsorption step, but this additional material could not be purified to high specific activity by column chromatography on IRC-50 as described. ACTH was prepared a total of four times by the procedures described here with reproducible results. The yield of final material varied from 50 to 60 mg. per kg. wet weight with a specific activity of 100 U.S.P. units per mg. in each case.

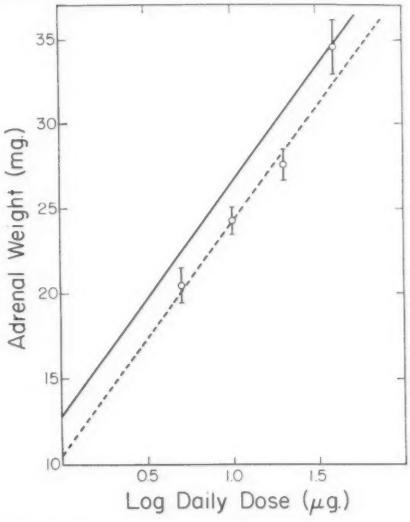

FIG. 1. Adrenal weight response of hypophysectomized male rats to IRC-50 column eluate. Each point represents data from 12 rats, the vertical lines indicating the standard errors.

Figure 1 shows the adrenal weight response in relation to dosage of the final product. The solid line is constructed from Li's equation for response to α-corticotropin of potency 150 U.S.P. units/mg. (2). The dotted line is calculated from the same equation for ACTH of potency of 100 U.S.P. units/mg. The response of the preparation corresponds closely to this cal-

culated line. The adrenal weights were significantly increased by 5 μg. per day for four days. This same dosage initiated repair of adrenal morphology as judged by increase of width of cortex, by finer lipid droplets with more uniform distribution, by reduction of the subglomerular lipid-free zone, and partial reconstitution of the zona reticularis.

Table 1 shows the efficacy of the product in stimulating calorigenesis and erythropoiesis in hypophysectomized rats. Doses above 10 μg. per day were required to cause a significant increase in calorigenesis, adrenal weight and red cell volume, and a decrease in thymus weight. Complete reinstatement of the deficiency in calorigenesis and red cell volume of the hypophysectomized rats was obtained with doses of 50 μg. and 100 μg. per day. No weight loss of these ACTH-injected animals occurred. The stimulation to calori-

TABLE 1. CALORIGENIC AND ERYTHROPOIETIC RESPONSES OF HYPOPHYSECTOMIZED RATS TO IRC-50 COLUMN PRODUCT*

Daily dose, μg.	Body weight, gm.		Hematocrit %	Red cell volume, ml./ gm. BW 100	Adrenals, mg.	Thymus, mg.	Metabolic rate, Cal./ m.²/hr.
	Initial	Final					
0	74	78	30.1 ± 0.6**	1.35 ± 0.02	6 ± 0	107 ± 5	19.5 ± 0.8
5	72	81	32.0 ± 1.1	1.41 ± 0.08	7 ± 1	88 ± 8	16.7 ± 0.7
10	76	86	32.7 ± 0.9	1.49 ± 0.07	9 ± 1	100 ± 9	21.1 ± 0.6
25	75	83	43.7 ± 1.4	2.11 ± 0.09	16 ± 2	< 10	27.0 ± 1.2
50	75	86	47.6 ± 3.3	2.39 ± 0.15	23 ± 3	< 10	30.7 ± 1.1
100	75	82	47.9 ± 1.3	2.43 ± 0.13	30 ± 3	< 10	32.2 ± 0.7
Normal (same age)		195	43.5 ± 0.5	2.42 ± 0.09	51 ± 3	243 ± 14	33.5 ± 1.1

* 6 rats per group.
** Figures preceded by ± are standard errors of the means.

genesis was not due to contamination of the ACTH with thyrotrophic hormone. The ACTH was assayed for thyrotrophic hormone activity in hypophysectomized rats maintained on low iodide diet by determining I^{131} uptake of the thyroids of the injected animals. No TSH activity was detectable in a four-day test at a total dosage of 3 mg. administered in saline or 400 μg. administered in the beeswax-peanut oil vehicle, nor in a fourteen day test at a total dosage of 1 mg. administered in beeswax.

DISCUSSION

Of the extraction methods used to obtain ACTH from the pituitary, aqueous extraction has usually been reported to be inefficient (8, 9). Extraction with acidified acetone has been used very successfully (10, 11, 12) as has extraction with hot glacial acetic acid (13). As reported here 0.1N HCl in water is an adequate extractant of ACTH from sheep pituitaries and yields a solution which can be handled simply and effectively for further purification of the hormone. Necessity for the use of the very large volumes of acetone or of glacial acetic acid is thus obviated. Heating of the aqueous extract removes the bulk of the protein. (This coagulation proce-

dure was adapted from a method used by van Dyke (14) for the preparation of a pituitary erythropoietic stimulant, which in turn was based on the method of Borsook (15) for the preparation of plasma erythropoietin. Collip, Anderson and Thompson (16) had much earlier shown that pituitary extracts boiled for 30 minutes still possessed adrenocorticotropic activity as shown by adrenal repair in hypophysectomized rats.) The oxycellulose adsorption method of Astwood *et al.* (17) was applied directly to the supernatant after heating. Column chromatography of ACTH on IRC-50, originally introduced by Dixon (18), was used here under conditions essentially those of Li *et al.* (19). At two stages in the procedure for preparation of ACTH described here (oxycellulose eluate and IRC-50 column eluate), solutions of the hormone at pH 6 were dialyzed against water for considerable periods of time. In the case of the IRC-50 column eluate the duration of dialysis was crucial. This dialysis separates a large quantity of salt from a relatively small weight of polypeptide hormone;[2] if inadequate time is allowed for dialysis the product will have a low specific activity while if dialysis is too prolonged, loss of ACTH occurs. It has been shown by several groups of workers including Tyslowitz (20), Geschwind *et al.* (21), and Cortis-Jones *et al.* (22) that ACTH passes through cellophane membranes. The results of the latter workers indicate that this effect is minimal at pH 6.

The method described here represents a simplification of existing methods for the purification of ACTH. Four kg. of glands were easily extracted at one time and the succeeding steps were carried out on the product from this total starting material. The yield of 202 mg. product from IRC-50 chromatography represents an excellent yield from 4 kg. wet weight of glands.

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[2] Since completing this work a method of Dixon (23) for freeing ACTH of salt has been called to our attention. It is likely that the Dixon method could be substituted for the dialysis procedure described in the present paper, though it should be emphasized that dialysis as described here allows recovery of the ACTH.

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STUDIES ON THE STATE OF INSULIN IN BLOOD: THE STATE AND TRANSPORT OF INSULIN IN BLOOD[1]

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ABSTRACT

Studies presented in this communication suggest that a major fraction of insulin in blood circulates in a complex form with other substances, more specifically, basic proteins. The basic protein-insulin complex(es) in blood could be adsorbed on cationic exchange resin, in the sodium form, and could be eluted from the resin by acid or alkali. Control experiments demonstrated that under identical conditions free, crystalline insulin could not be adsorbed on this resin.

Blood insulin in its complex form did not exhibit full insulin activity when examined by *in vitro* assays. Full insulin activity could be demonstrated by dissociation of the insulin from its complex(es) either through isoelectric precipitation of the carrier protein(s) or by incubation of the insulin complex(es) with human or rat adipose tissue extracts.

The transport of insulin in blood by basic proteins may serve as a regulatory mechanism of insulin activity through the equilibrium between "bound" and "unbound" insulin.

INTRODUCTION

EARLIER reports by the author and collaborators (1–5) suggested that insulin, measured as insulin-like activity, in human blood and in human and bovine pancreas is in a complex form with other substances, more specifically, basic proteins. The blood and pancreatic insulin complexes could be adsorbed on cationic exchange resin (Na^+ cycle) and could be eluted from the resin by acid or alkali. Control experiments demonstrated that under identical conditions free, crystalline insulin could not be adsorbed on this resin.

There is evidence suggesting that the blood insulin complexes eluted from the resin by acid or alkali did not exhibit full insulin-like activity (6) when they were examined *in vitro* by the rat diaphragm tissue assay, as employed in these studies (7). Increased insulin-like activity was demonstrated upon centrifugation of the resin eluates at pH 9.8 (6) or by incubation of the resin eluates with rat or human adipose tissue extracts (8).

Received April 21, 1960.

[1] These studies were supported in part by the National Foundation, and the Eli Lilly Company. The blood serving as a source for the insulin and basic-protein-insulin complexes studied was collected and processed under a grant (H-2929) from National Heart Institute.

These observations were made under conditions specified in this communication and elsewhere (6, 8). The results may differ when other methods of insulin assay are employed since different tissues, or even the same tissue handled differently, may utilize the insulin from its complexed form.

The present communication describes in detail studies concerned with the state of insulin in blood and methods employed for the extraction and purification of the insulin complex(es).

MATERIALS AND METHODS

Preparation of the resin: Two cationic exchange resins were primarily employed in these studies: Dowex[2]-50×8 (mesh 25–50) and Dowex-50×2 resin (mesh 50–100). The resin was converted to the sodium form by the procedure described below, which is standard method for the preparation of the resin Dowex-50×8 for routine blood collection. The introduction of cationic exchange resin (Na^+ cycle) for blood collection is based on the exchange of blood Ca^{++} for 2 Na^+ of the resin (4). The removal of Ca^{++} from blood by the resin prevents blood coagulation and provides a method for blood collection without the use of anticoagulants.

Procedure: One liter of wet resin on the H^+ cycle is placed in a Buchner funnel and is washed with 6 liters of distilled water.

The resin is washed with 3 liters of 6 M sodium chloride solution, followed by washing with 3 liters of distilled water. The pH should be about 2.7. The resin is washed with one liter of M/20 $Na_2HPO_4 \cdot 7H_2O$ (Bakers) solution followed by washing with 3 liters of 0.15 M sodium chloride. The final pH should be 6.6 ± 0.4.

The pH of the resin is determined as follows: Approximately 1 ml. of resin beads is equilibrated with 5 ml. of M/100 sodium sulfate. The resin beads are allowed to settle, and the pH is measured on the supernatant solution.

Assays for insulin-like activity: The *in vivo* assay for insulin-like activity employed in these studies utilizes the hypophysectomized-alloxanized rat and is based upon the diminishing increment of blood glucose, which occurs in response to insulin injection, following gavage with the polysaccharide dextrin (9).

In vitro assays: (a) *Rat adipose tissue assay:* This assay is based upon the effect of insulin on the production of $C^{14}O_2$ from glucose-C^{14} by this tissue (10). (b) *Rat diaphragm tissue assay:* The rat diaphragm tissue assay is based on the increment in glucose uptake by this tissue in the presence of insulin (7).

Insulin-I^{131} employed in these studies was obtained from the Abbott Laboratories, Oak Ridge, Tennessee, and had a specific activity of 5 to 10 mc. per mg. of insulin.

Paper electrophoresis runs were carried out on the Spinco model R at pH 7.6 (phosphate buffer, ionic strength 0.1). The material was applied on 4 by 31 cm. S and S 470 paper strips and was run for 16 hours at 2° C and 40 ma.

Adsorption of blood insulin-like activity on the resin (Na^+ cycle): Whole blood or serum is passed through a column containing Dowex-50 resin (Na^+ cycle) at a rate of 20 ml. per minute. Approximately 180 ml. of Dowex-50 resin are used per liter of blood or serum. In routine blood collection 500 ml. of whole blood are collected through a column (10×3.5 cm.) containing about 90 ml. of wet resin.

Following the treatment of blood or serum with the resin, the column is washed repeatedly with cold 0.15 M sodium chloride solution in order to remove the contaminating proteins (Fig. 1).

Elution of insulin-like activity from the resin: The elution of insulin-like activity from the resin has been accomplished with either one of the following procedures:

[2] Dow Chemical Company, Midland, Michigan.

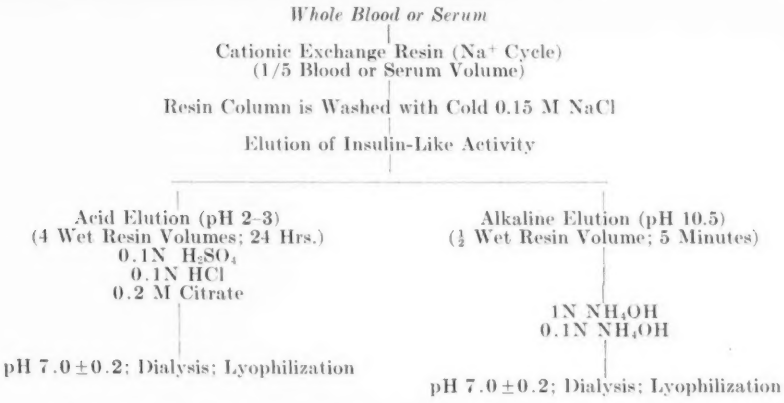
Whole Blood or Serum
Cationic Exchange Resin (Na^+ Cycle)
(1/5 Blood or Serum Volume)
Resin Column is Washed with Cold 0.15 M NaCl
Elution of Insulin-Like Activity
Acid Elution (pH 2–3)
(4 Wet Resin Volumes; 24 Hrs.)
0.1N H_2SO_4
0.1N HCl
0.2 M Citrate
pH 7.0±0.2; Dialysis; Lyophilization
Alkaline Elution (pH 10.5)
(½ Wet Resin Volume; 5 Minutes)
1N NH_4OH
0.1N NH_4OH
pH 7.0±0.2; Dialysis; Lyophilization

FIG. 1. Extraction of insulin-like activity from human blood or serum.

Acid elution: Acid solutions such as 0.1N H_2SO_4, 0.1N HCl or 0.2 M citric acid have been employed for the elution of insulin at pH 2.0. The resin, following blood or serum treatment, was washed with 0.15 M sodium chloride solution, eluted two times, using each time two-resin volumes of acid for 12 hours at 2° C, and finally washed with one-resin volume of cold distilled water. The combined eluates and washes were brought to pH 7.0±0.2, then dialyzed overnight against ten volumes of 0.02 M sodium chloride solution and finally lyophilized. The dry powder was collected and stored at 2° C or dissolved in the minimum possible volume of distilled water and stored at −20° C.

Alkali elution: The resin, following washing with cold 0.15 M sodium chloride solution, was eluted with ½ resin volume of ammonium hydroxide (0.1 M or 1 M). The alkaline effluent was collected in ¼ resin volume of 0.1N H_2SO_4 (pH 2.5) in order to avoid a long exposure of the insulin solution at high pH. Elution with alkali should be accomplished within 5 minutes. The resin, following alkali elution, was washed with ½ resin volume of distilled water, and the pH of the combined eluates and washes was adjusted to 7.0±0.2. The combined eluates and washes were dialyzed overnight at 2° C against 0.02 M sodium chloride and finally lyophilized.

RESULTS

Insulin-complexes in blood: The results obtained with the methods described are summarized in Tables 1 and 2. The various lots of resin eluates were from pooled samples obtained from four to fifty donors (1 to 10 liters of blood or serum).

The data show that the amount of insulin in the resin eluates depends on the individual insulin assay, on the conditions of the resin eluates with regard to dialysis, the choice of the eluant, and the dissociation of the insulin complex(es). Treatment of the resin eluates either at pH 9.8 or by incubation with rat adipose tissue extracts releases a many-fold increase of insulin activity (Table 2).

These data suggested that the insulin in the resin eluates is present in a complexed form. Centrifugation of the resin eluates at pH 9.8 (which removes the basic-protein(s), leaving the insulin in the supernatant fluid) or

TABLE 1. INSULIN-LIKE ACTIVITY OF RESIN ELUATES

Lot No. Blood (B) Serum (S)	Eluant	Dialysis Dialyzed (D) Undialyzed (UN)	Rat adipose tissue assay Insulin in		Rat diaphragm tissue assay Insulin in	
			mu per mg. nitrogen	mu per liter of plasma	mu per mg. nitrogen	mu per liter of plasma
011 (B)	0.2 M citrate; pH 3.0	(D)	—	—	—	120
016 (B)	0.1 N H_2SO_4; pH 2.0	(D)	7	45	0	0
025 (S)	0.2 M citrate; pH 2.0	(D)	30	150	6	50
028 (S)	0.1 N HCl; pH 2.0	(UN)	26	80	—	—
052 (B)	0.1 N NH_4OH; pH 10.5	(D)	1.4	20	0	0
057 (B)	1 N NH_4OH; pH 11.0	(D)	13.8	23	6.2	10
071 (B)	0.1 N NH_4OH; pH 10.5	(UN)	—	—	2.5	80
072 (B)	1 N NH_4OH; pH 11.0	(UN)	—	—	2.7	80
080 (B)	0.1 N NH_4OH; pH 11.0	(D)	—	—	1	7.5
073 (B)	1 N NH_4OH; pH 11.5	(UN)	—	—	16	55
101* (B)	0.1 N NH_4OH; pH 10.5 + 1 N NH_4OH; pH 11.0	(D)	—	—	0.44	7.2

* Lot 101 represents pooled eluates prepared both with 0.1 N and 1 N NH_4OH.

incubation at 37° C with adipose tissue extracts, liberated the insulin from its complex, resulting in an increased insulin activity.

The insulin activity of the "untreated" resin eluates (Table 1) depends, as indicated above, on the choice of eluant and the dialysis of the resin eluates prior to insulin assay. Citrate eluates yielded higher insulin values, which suggests that citrate removes part of the insulin-binding substances by forming insoluble complexes with them when eluting with citrate at pH 2–3.

Dialysis of the resin eluates, following acid or alkali elution, also affects the insulin activity (Table 1). Undialyzed samples diluted 5 to 10 times

TABLE 2. INSULIN-LIKE ACTIVITY OF RESIN ELUATES FOLLOWING CENTRIFUGATION AT pH 9.8* OR INCUBATION WITH ADIPOSE TISSUE EXTRACTS†

Lot No. Blood (B) Serum (S)	Eluant	Rat diaphragm tissue assay: Without treatment Insulin in		Centrifugation at pH 9.8* Insulin in		Incubation with adipose tissue extracts† Insulin in	
		mu per mg. nitrogen	mu per liter of plasma	mu per mg. nitrogen	mu per liter of plasma	mu per mg. nitrogen	mu per liter of plasma
016 (B)	0.1 N H_2SO_4; pH 2.0	0	0	7	35	35	225
052 (B)	0.1 N NH_4OH; pH 10.5	0	0	5	51	—	—
080 (B)	0.1 N NH_4OH; pH 11.0	1	7.5	—	—	20	150
081 (B)	0.1 N NH_4OH; pH 10.5	0	0	—	—	5	120
101 (B)	0.1 N NH_4OH; pH 10.5 + 1 N NH_4OH; pH 11.0	0.44	7.2	—	—	11	180

* Assayed for insulin-like activity following centrifugation of the eluates at pH 9.8.
† Assayed for insulin-like activity following incubation of the eluates with human or rat adipose tissue extracts.

with Gey and Gey bicarbonate buffer showed higher insulin levels than samples prepared similarly and assayed after being dialyzed for 12 to 48 hours. These observations indicate that the insulin complexes, which are dissociated during acid or alkali elution, may recombine slowly at neutral pH. Undialyzed samples therefore contain a larger portion of "free" insulin, whereas, during the dialysis of resin eluates, insulin recombines with its binding substances, resulting in reduction or disappearance of insulin activity when tested with the rat diaphragm tissue assay. The insulin activity may be recovered, as described above, upon dissociation of the in-

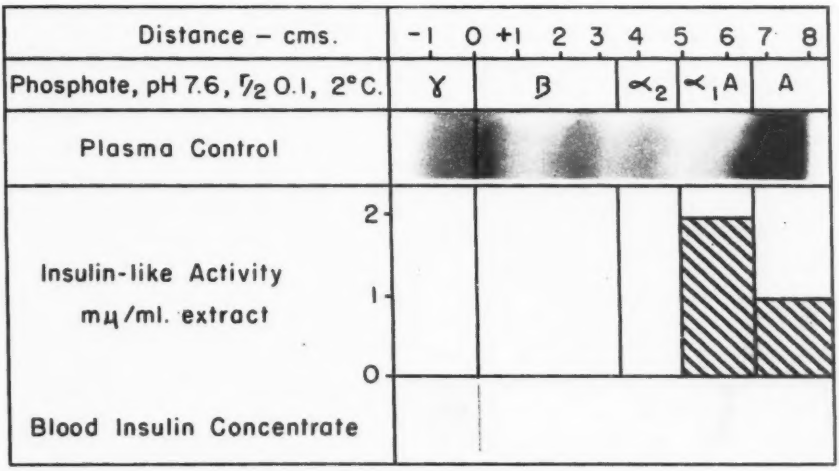

FIG. 2. Paper electrophoresis of blood insulin concentrate* (citrate eluates†).

* The insulin concentrate and the plasma control were applied on paper strips (S and S 470), subjected to electrophoresis for 16 hours at 40 ma. The paper strip carrying the plasma control was developed, and the paper strips carrying the insulin concentrates (duplicate experiment) were cut into five sections: The albumin (A), $A\alpha_1$-globulin, α_2-globulin, β-globulin and γ-globulin. The proteins were extracted from each paper cut with 15 ml. of Krebs' bicarbonate buffer and were assayed for insulin activity by the rat adipose tissue assay (10).

† It is suggested that the insulin in the citrate eluates is in the "free" state, the insulin-binding basic proteins having formed insoluble complexes with the citrate.

sulin from its complex(es) with centrifugation at pH 9.8 or by incubating the eluates with rat adipose tissue extracts (Fig. 2).

Cationic character of insulin-binding substances: Insulin at physiological pH is a negatively charged molecule. Human blood insulin, liberated from its complex during the elution of the resin with citrate (pH 2.0), migrates on paper electrophoresis (pH 7.6) as an anion (Fig. 2). The adsorption of blood insulin on a basic exchange resin therefore suggests that insulin in blood is transported as a complex with other substances of higher isoelectric points (1–2), possibly basic proteins, the insulin complex being adsorbed to the resin. A diagrammatic representation of this supposition is

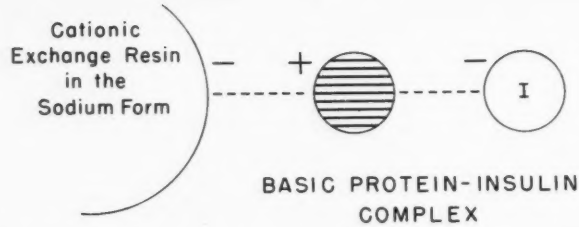

FIG. 3. Diagrammatic representation of the adsorption of the blood basic-protein-insulin complex on the resin.

presented in Figure 3, indicating that blood insulin is adsorbed on the resin "indirectly" through the binding of its cationic carrier.

Insulin-binding basic protein(s): Dialysis of resin eluates at acid (pH 2.0–3.0) or neutral pH for 12 to 48 hours did not remove the insulin-binding substances. This observation suggested that these substances, which at low pH dissociate from insulin, are micromolecules, possibly of a protein nature. Concentrates of the insulin-binding substances were prepared from resin eluates by precipitation at pH 9.8 and removal of the precipitate by centrifugation. Such precipitates from resin eluates, equivalent to about 40 liters of plasma, were pooled and dissolved in 0.5 ml. of acetate buffer (0.1 M), pH 4.0. A sample of this preparation, equivalent to about 10 liters of original plasma, was applied on paper strips (S and S 470) and was subjected to electrophoresis in phosphate buffer ($\Gamma/2$ 0.1, pH 7.4) for 16 hours at 25° C and at 40 ma. Figure 4 shows the presence of a protein on the paper strip, stained with ninhydrin, having a large basic net charge.

These studies suggest that the substances binding the insulin in blood

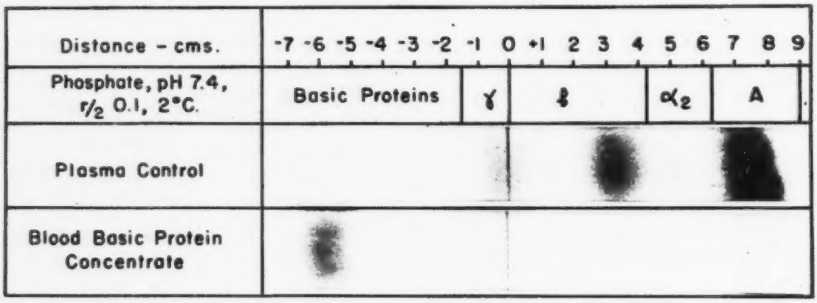

FIG. 4. Paper electrophoresis of blood basic-protein(s) concentrate* prepared with the use of cationic exchange resin (Na^+ cycle).

* Concentrates of the insulin-binding basic protein(s) were prepared from resin eluates by precipitation at pH 9.8 and removing the precipitate by centrifugation. Such precipitates from resin eluates, equivalent to about 40 liters of plasma, were pooled and dissolved in 0.5 ml. of acetate buffer (0.1 M) pH 4.0.

TABLE 3. INSULIN-I^{131}* ADDED *in vitro* TO UNDILUTED BLOOD SERUM† AND TREATED WITH CATIONIC EXCHANGE RESIN (Na^+ FORM)

Before resin treatment c/m/ml. serum	After resin treatment c/m/ml. serum
86,207	79,365
22,000	21,000
60,250	60,080
84,034	83,894
80,654	80,510
86,207	79,636
12,305	12,202
5,005	5,000
2,128	2,100

* The amount of Insulin-I^{131} added to serum varied from 5 $\mu\mu$ to 200 $\mu\mu$ per ml. of undiluted serum.
† Incubated at 2° C and 25° C for 5 minutes and 24 hours.
Blood serum volume: 50 to 100 ml.

are proteins with a strongly basic character, which explains the adsorption of the complex on the base exchange resin. The insulin-binding protein(s) in blood were therefore designated as the blood insulin-binding basic proteins, the word "basic" being used to show the cationic character of the protein(s). Similar insulin complexes have been identified in human and bovine pancreas (3), and the word "blood" or "pancreas" should be used to define the origin of the various preparations.

Specificity of the blood-basic protein insulin complex(es): At the present time it is not known whether the presence of the insulin complex in blood is the result of non-specific interaction of insulin and basic proteins or whether the insulin complex is specific, being generated in certain specific sites of the body.

From the data in Tables 3 and 4 it appears that there is no excess of "free" insulin-binding basic proteins in the blood stream. Insulin-I^{131} added *in vitro* to undiluted serum or administered intravenously to non-diabetic subjects was not adsorbed on the cationic exchange resin (Na^+ cycle). It is possible that the added insulin-I^{131} was bound by blood proteins other than the insulin-binding basic proteins. The criteria for the insulin-

TABLE 4. INSULIN-I^{131} INJECTED INTRAVENOUSLY TO NON-DIABETIC SUBJECTS*

Experiment	Blood simples collected after I.V. injection	Before resin treatment (c/m/ml. serum)	After resin treatment (c/m/ml. serum)
I	2 minutes	16,188	14,791
	20 minutes	829	740
II	2 minutes	46,250	45,620
	35 minutes	8,380	7,820
	65 minutes	7,525	7,000

* Blood samples were collected at various intervals after the I.V. administration and the blood serum was treated with cationic exchange resin (Na^+ form). The blood samples, following I.V. administration of insulin-I^{131}, were made available through the courtesy and generosity of Dr. Marvin L. Mitchell.

binding basic protein are both its ability to bind insulin and its adsorption on cationic exchange resin (Na^+ cycle). One may suggest that there is a "stoichiometric" relationship between the basic protein and the insulin. The presence of only trace amounts of basic protein(s) in the resin eluates (Fig. 4) is in accordance with this suggestion.

DISCUSSION

The methods of insulin-like extraction from blood described in this communication are simple and do not waste other blood or serum components. When blood is collected over cationic exchange resin to prevent coagulation, the resin is discarded, providing a convenient source for the concentration and purification of insulin from large volumes of blood or serum.

The high degree of purification of the insulin-like activity present in the resin eluates reduces the possibility of the presence of other serum components interfering with the assay of insulin-like activity. It is suggested by the following observations that the insulin-like activity present in the eluates represents true insulin activity: (1) The insulin-like activity has been identified by three different assays, *in vivo* and *in vitro;* (2) Insulin-like activity in the resin eluates has been determined in dilutions of 50- to 100-fold, making it most unlikely that non-specific factors interfere in the determinations; (3) Anti-insulin antibodies neutralized the "free" insulin activity of the eluates, suggesting a specific reaction of the antibody with the blood insulin.[3] Nevertheless it is conceivable that non-insulin-hypoglycemic factor(s) may be present in this system.

Prolonged acid elution or elution for a few minutes with 0.1N NH_4OH produces similar results. The use of 0.1N NH_4OH for the elution of the insulin-like activity is the most convenient method because of the short time of elution required (5 minutes), the low salt concentration and the small volumes of eluates as compared with the acid elution. Losses of insulin-like activity during the elution of the resin with alkali may be caused by pH values in excess of 11.0. The pH should be controlled carefully during alkali elution, and the pH of the eluates should not exceed 10.5 to 11.1 during the short period of time required for the elution.

Citrate eluates in three different cases provided higher insulin values than eluates obtained with sulfuric acid or ammonium hydroxide when they were examined for insulin-like activity (Table 1). This observation suggests that the basic protein-citrate salts, which are formed during the elution of the resin with citrate, are less soluble than those of the basic protein-sulfate salts. The insoluble citrate complexes are removed from the system by centrifugation of the eluates at pH 3.0, resulting in a larger portion of the insulin in the "free" state. The finding that the insulin activity of citrate eluates subjected to paper electrophoresis at pH 7.6 migrates in

[3] Unpublished data obtained by Dr. H. Narahara.

the A/a_1-globulin zone, i.e. with the mobility of free insulin, corroborates the suggestion that citrate eluates may be rich in free insulin. However, in the paper electrophoresis experiment the eluates, representing 30 to 40 liters of plasma, were repeatedly dialyzed and lyophilized in order to reduce the volume of the eluates. Dissociation of the insulin from its complex also may be due to the manipulation of this preparation.

On paper electrophoresis the basic protein migrates as a single cationic protein. However, other criteria of purity are necessary in order to establish homogeneity of this preparation. The protein does not pass through a cellophane membrane at pH 3.0 or 7.0, which may indicate a relatively large molecular weight. From comparative paper electrophoresis studies with the bovine pancreatic insulin-binding basic protein it appears that the human blood preparation is more basic, as indicated by its faster migration on paper. Its isoelectric point may be close to pH 10.0 since at this pH the basic protein precipitates and may be removed by centrifugation. The yield of the blood-basic protein from about 40 liters of plasma was extremely low, and an amount equivalent to 10 liters of plasma was applied on one single paper strip (Fig. 4). The protein of the paper was stained only by ninhydrine and not by bromphenol blue.

The transport of insulin by proteins of higher isoelectric point may serve as a means of regulation of insulin activity in the blood stream. Such a regulatory mechanism is probably based on the release of insulin in blood from its complex through a physicochemical equilibrium. The rate of dissociation of this complex may be affected by enzymes. Direct evidence for enzyme intervention *in vivo* is lacking. The finding, however, that blood and pancreatic insulin complexes are dissociated *in vitro* at physiological pH through enzyme intervention (8) is suggestive.

The observation, that a many-fold increase of insulin activity occurs in the resin eluates upon dissociation of the insulin complex, stimulates the question as to what are the "true" levels of insulin in blood. It is possible to suggest that various current insulin assays determine only a fraction of the "total" insulin activity in serum.

The experiment presented in Table 4 showed that when exogenous insulin is injected intravenously, no insulin binding protein appears in blood to bind it. One may suggest that the complex is formed *in vivo*, perhaps in the pancreas. However, in interpreting the results of this experiment, it should be noted that the insulin employed for intravenous administration was iodinated insulin prepared from bovine and porcine pancreas.

The whole blood and serum employed in these studies were collected from non-diabetic subjects. Preliminary studies indicate that identical or similar complexes are present in the blood of diabetics and of diabetics with insulin resistance. These complexes differ from the antibody-insulin complexes (11) previously described in the blood of diabetics who had been under insulin therapy for a long period of time. The difference was demon-

strated by showing that the antibody-insulin complexes are not adsorbed on cationic exchange resin (Na^+ cycle).

Similar observations, indicating the presence of basic-protein-insulin complexes have been made upon the pancreatic tissue of diabetic subjects. The question of "utilization" of the stored insulin in blood and pancreas of diabetics arises from these observations, particularly in connection with the mechanism of dissociation of the insulin complexes *in vivo*.

Acknowledgments

I gratefully wish to express my appreciation to Dr. A. E. Renold and his associates, Drs. D. Martin, Y. M. Dagenais and J. Steinke, for the bioassays carried out by the rat adipose tissue assay, to Drs. K. Gundersen, H. Narahara and P. Randle for the rat diaphragm tissue assays, and to Dr. P. Beigelman for the *in vivo* assays. My thanks to Mr. Anatolii Echardt for his helpful assistance throughout this work.

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ANTI-ESTROGEN ASSAY OF NEUTRAL STEROIDS ADMINISTERED BY SUBCUTANEOUS INJECTION[1,2]

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ABSTRACT

A method for the evaluation of anti-estrogenic activity is described using 20–22-day old estrone stimulated female Swiss albino mice. Both the estrone and the test compound were injected subcutaneously but at separate sites. The end-point was the degree of inhibition of estrogen-produced uterine growth. The relative antiestrogenic potency of 26 steroids is reported on the basis of the minimum weight of compound needed to produce a statistically significant inhibition of the uterine stimulation produced by 0.4 μg. of estrone. Correlations of structure and activity may be summarized as follows: reduction of a Δ^4-3-keto group in ring A to a 5α-3-keto grouping produced an enhanced activity in testosterone but not in three other steroids studied; formation of the 19-nor steroid led to enormous increases in activity for 17α-ethyltestosterone (62 times) and 17-ethynyltestosterone (31 times) but not in four other steroids of varied structures; and substitution of 17α-hydrogen by a methyl, ethyl, or ethynyl group usually produces a compound with increased anti-estrogenic activity.

THE fact that various steroids may inhibit the action of estrogens has been described by many investigators since the work of De Fremery *et al.* (1), who demonstrated that the subcutaneous injection of progesterone inhibited the vaginal cornification in ovariectomized mice receiving an impure estrogen preparation by mouth. This phenomenon was also observed by Allen and Meyer (2). Robson (3), in a detailed study, demonstrated that the vaginal cornification reaction, due either to estrone or estradiol-17β in ovariectomized mice, may be inhibited by the simultaneous administration of testosterone, progesterone, androstane-3α,17β-diol and Δ^4-androstene-3,17-dione. The results of these studies may be questioned, since the estrogen and inhibitor were contained in the same solu-

Received April 21, 1960.

[1] Supported in part by Grant No. CY-2193 from the U. S. Public Health Service.

[2] This is Steroids CLI in the series published from the Syntex Research Laboratories.

tion. The relatively large quantity of test compound could possibly prevent the estrogen from being absorbed at the rate that the estrogen was absorbed when administered alone.

Among the three estrogens, estrone, estriol, and estradiol-17β, a curious interaction has been demonstrated in which one estrogen may limit the biological effectiveness of the other estrogens (4). These interactions are not limited to estrone, estriol, and estradiol-17β. Huggins and Jensen (5) have reported that when the hypophysectomized female rats were treated with estrone, the usual uterine hypertrophy could be inhibited by a variety of estrogens, including 16-epiestriol, 17-epiestriol, estradiol-16α, estradiol--16β, 6-ketoestrone, and 6-ketoestradiol-17β.

The inhibition of estrogens by corticoids has been reported (6, 7, 8, 9). Velardo (10) has summarized his extensive findings using the uterus of the ovariectomized mouse as the end-point. This group confirmed the finding that progesterone causes a significant inhibition of estrogens at low doses but synergizes with estradiol-17β when given in doses above 2.5 mg. daily. Testosterone is a highly effective anti-estrogen. Corticoids are effective and the order of activity may be summarized as 9α-fluoroprednisolone > 9α-fluorocortisol > cortisol > cortisone > prednisone > prednisolone > desoxycorticosterone. This order of effectiveness does not correlate with other activities of corticoids.

Recently a group of papers have appeared dealing with the anti-estrogenic activity of certain synthetic steroids which show intense activity (11, 12, 13, 14, 15). Edgren *et al.* (15) reported that some 17α-alkyl derivatives are extremely active, that the nature of the alkyl group has an important effect on the potency, and that the dihydro-19-nortestosterone as well as the 5(10) dehydro isomer are much less active than the Δ^4 steroids. The stimulating and suppressing compounds were injected as a single solution at a single site.

Certain synthetic compounds such as (Di-*p*-hydroxyphenyl)-butane; -pentane; -hexane; and -1,4-pentanedien-3-one inhibit the proliferation and cornification in the vaginal epithelium of rats treated subcutaneously with estradiol benzoate (16). Villee (17, 18) has shown that certain anti-estrogenic activity may be detected with an *in vitro* test employing the estrogen sensitive enzyme, isocitric acid dehydrogenase, derived from human placental tissue. Emmens and Cox (19) have demonstrated that dimethylstilbestrol, a pro-estrogen, inhibits the response of estrogens when the two substances are administered intravaginally to spayed mice. In a second paper Emmens *et al.* (20) found this type of activity for ethylstilbestrol and n-propylstilbestrol. These stilbestrol derivatives inhibit the increase in vaginal mitosis and epithelial thickness produced by the estrogens whereas testosterone and progesterone, even in high concentration, inhibited cornification but not mitosis.

Most studies with anti-estrogenic substances have indicated only a

partial suppression of stimulatory activity of the estrogen employed. With the synthetic substance 1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-methoxyl ethanol (Mer-25), a suppression of almost 95% has been effected. This substance does produce some stimulation of the uterus. The anti-estrogenic action has been demonstrated in the vaginal epithelium and the uterus (21).

This communication presents a convenient, simple method for the determination of the anti-estrogenic action of steroids, the relative potency of a variety of new steroids, and finally describes the influence of structural changes on activity.

EXPERIMENTAL

Twenty- to twenty-two-day old Swiss albino mice were injected subcutaneously once daily with estrone for three days. The total dose of 0.4 μg. was contained in 0.3 ml. of sesame oil and 0.1 ml. was injected daily. Control groups of mice received only sesame oil. The test substance was injected subcutaneously daily in 0.2 ml. of aqueous suspending vehicle at a different site from that used for the estrone injections. Separate sites were used for the injection of the estrogen and the test compound to prevent the possibility that a lowered estrogenic effect might be due to interference of estrogen absorption from the injection site. The test compounds were administered in an aqueous suspending medium since many of these substances at the dose levels studied were insoluble in oil. The aqueous suspending medium consisted of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethylcellulose (0.5%), and benzyl alcohol (0.9%). One day after the last injections the animals were sacrificed and the uterine weights and body weights determined.

The assays were usually performed in groups of 132 mice which were divided into 14 groups. One group of 12 mice served as sesame oil injected control animals, another group of 12 mice received a total dose of 0.4 μg. estrone. The remaining 12 groups of 9 mice each received the test compounds. Usually a single compound was studied at three or more concentrations in any specific assay. Finally, to arrive at the relative activity of a given compound, all the data for a specific compound at a specific dose were combined. To determine the minimum dose to produce inhibition, a response was chosen which, when calculated statistically, gave a value of P of less than 0.02.

RESULTS

The relative anti-estrogenic potency of various steroids is listed in Table 1, which summarizes the information on each of the compounds studied. Included are the total number of mice, the dosage range, the minimum dose which showed a statistically significant inhibition of estrogen stimulated uterine growth, and the maximum inhibition attained with any dose studied. The compounds are arranged in decreasing order of activity on the basis of amount of material needed to produce an inhibitory effect.

The inhibitory activity of testosterone was enhanced five times by reduction to the dihydro 5α derivative (Table 2). Similar reduction of 17α-methyl-19-nortestosterone resulted in a 50% reduction in activity, while the same chemical change in 17α-ethynyl-19-nortestosterone yielded a compound possessing only 3% of the original activity. Reduction of 19-nor-

TABLE 1. RELATIVE ANTI-ESTROGENIC ACTIVITIES OF VARIOUS STEROIDS (INJECTION)

Steroid	Total No. of mice	Dosage range studied, μg.	Minimum dose to produce inhibition, μg.	Maximum inhibition %
2α,17α-Dimethyl-17β-Hydroxyandrostan-3-one	81	2–4000	2	30
17α-Ethyl-19-Nortestosterone	186	2–4000	8	54
17α-Ethynyl-19-Nortestosterone	180	1–4000	16	56
17α-Methyl-19-Nortestosterone	126	2–1000	32	46
17α-Methyltestosterone	135	32–4000	32	35
2α-Methyl-17β-Hydroxyandrostan-3-one	90	1–1000	50	47
17α-Methyl-19-Nor-17β-Hydroxyandrostan-3-one	215	1–4000	50	38
17β-Hydroxyandrostan-3-one	126	15–4000	100	30
17α-Ethyl-19-Nor-17β-Hydroxyandrostan-3-one	171	1–5000	250	27
Androstane-3β,17β-diol	140	2–4000	250	22
Testosterone	99	64–4000	500	36
19-Nor-17β-Hydroxyandrostan-3-one	135	1–4000	500	32
19-Nortestosterone	135	1–4000	500	33
Progesterone	129	10–4000	500	56
17α-Ethynyl-19-Nor-17β-Hydroxyandrostan-3-one	90	10–4000	500	27
17α-Vinyl-19-Nor-17β-Hydroxyandrostan-3-one	126	64–4000	500	20
17α-Ethynyltestosterone	114	8–2000	500	22
17α-Ethynyl-19-Norandrostane-3β,17β-diol	137	10–4000	1000	14
19-Norandrostane-3β,17β-diol	72	1000–4000	4000 No response	—
17α-Methyl-19-Norandrostane-3β,17β-diol	90	1000–4000	4000	23
17α-Ethyl-19-Norandrostane-3β,17β-diol	36	500–4000	4000 No response	—
17α-Vinyl-19-Norandrostane-3β,17β-diol	36	500–4000	4000 No response	—
Δ^5-Androstene-3β,17β-diol	72	10–4000	4000 No response	—

testosterone to the 5α dihydro derivative did not influence the anti-estrogenic activity.

Removal of the C-19 group resulted in all three logical possibilities; that is, increase, decrease, and no change in anti-estrogen activity (Table 3). The 19-nor derivatives of testosterone and 17α-methyltestosterone were equal in potency to that of the parent compound. Two compounds, androstane-3β,17β-diol and 17β-hydroxyandrostan-3-one, showed significant loss of activity on removal of carbon 19. Most dramatic was the difference between androstane-3β,17β-diol, which was active at 125 μg., while the corresponding 19-nor steroid was negative at 4000 μg. A 31- and 62-fold in-

TABLE 2. INFLUENCE OF REDUCTION OF Δ^1 GROUP TO 5α CONFIGURATION ON ANTI-ESTROGENIC ACTIVITY (INJECTION)

Steroid	No. of mice on steroid	No. of mice on 5α-steroid	Lowest total dose producing inhibition		5α-reducing enhancing ratio = A/B
			Steroid (A)	5α-steroid (B)	
Testosterone	99	126	500	100	5.0
19-Nortestosterone	135	135	500	500	1.0
17α-Methyl-19-Nortestosterone	126	153	32	64	0.5
17α-Ethynyl-19-Nortestosterone	153	90	16	500	0.03

TABLE 3. INFLUENCE OF LOSS OF C-19- (FORMATION 19-NOR DERIVATIVE) ON ANTI-ESTROGENIC ACTIVITY (INJECTION)

Steroid	No. of mice on steroid	No. of mice on 19-nor steroid	Lowest total dose (μg.) producing inhibition		19-nor enhancing ratio = A/B
			Steroid (A)	19-nor steroid (B)	
Testosterone	99	135	500	500	1.0
17α-Methyl-testosterone	135	126	32	32	1.00
Androstane-3β,17β-diol	117	72	125	4000	< 0.03
				Vegative	
17α-Ethynyl-testosterone	54	180	500	16	31.0
17α-Ethyl-testosterone	198	153	500	8	62
17β-Hydroxyandrostan-3-one	126	135	100	500	0.2

crease in activity resulted in the removal of carbon 19 from ethynyltestosterone and ethyltestosterone, respectively.

The substitution of a 17α-methyl group for the 17α-hydrogen atom significantly increased the anti-estrogenic activity in all instances except 17β-hydroxyandrostan-3-one, where a 5-fold decrease was found (Table 4). This enhancement in activity for the other compounds varied from >1 for 19-norandrostane-3β,17β-diol to 25 for the enhancement ratio for 2α-methyl-17β-hydroxyandrostan-3-one. The addition of the 17α-methyl group increased the activity of testosterone, 19-nor-17β-hydroxyandrostan-3-one, and 19-nortestosterone 7.8, 7.8, and 15.6 times, respectively. The introduction of the 17α-methyl into the Δ^5-androstane-3β,17β-diol molecule resulted in a compound active at 2000 μg., while the parent compound was inactive at twice this dose.

The data in Tables 5 and 6 are similar to those presented in Table 4 except for the nature of the 17α-alkyl group. The anti-estrogenic activity of testosterone was not modified by the 17α-ethyl or 17α-ethynyl group. A dose of 500 μg. of 19-nortestosterone was required to produce a statistically significant depression in the estrone stimulated uterus, whereas the

TABLE 4. INFLUENCE OF 17α-METHYL SUBSTITUENT ON ANTI-ESTROGENIC ACTIVITY (INJECTION)

Steroid	No. of mice on steroid	No. of mice on 17α-CH_3 steroid	Lowest total dose (μg.) producing inhibition		17α-CH_3 enhancing ratio
			Steroid	17α-CH_3 steroid	
Testosterone	99	135	500	32	15.6
19-Nortestosterone	135	126	500	32	15.6
19-Nor-17β-Hydroxyandrostan-3-one	135	153	500	64	7.8
17β-Hydroxyandrostan-3-one	126	220	100	500	0.2
2α-Methyl-17β-Hydroxyandrostan-3-one	90	81	50	2	26
Δ^5-Androstene-3β,17β-diol	72	220	4000	2000	> 2
			Negative		
19-Norandrostane-3β,17β-diol	72	90	4000	4000	> 1
			Negative		

TABLE 5. INFLUENCE OF 17α-ETHYL SUBSTITUENT ON ANTI-ESTROGENIC ACTIVITY (INJECTION)

Steroid	No. of mice on steroid	No. of mice on 17α-ethyl steroid	Lowest total dose (μg.) producing inhibition		17α-ethyl enhancing ratio = A/B
			Steroid (A)	17α-ethyl steroid (B)	
Testosterone	99	198	500	500	1.0
19-Nor-17β-Hydroxyandrostan-3-one	135	180	500	250	2.0
19-Nortestosterone	135	153	500	8	62
19-Norandrostane-3β,17β-diol	72	36	4000 Negative	4000 Negative	—

17α-ethyl derivative required only 8 μg. and the 17α-ethynyl derivative required 16 μg. The enhancement ratios are thus set at 62 and 31 for the ethyl and ethynyl derivatives, respectively. 19-Norandrostane-3β,17β-diol was inactive at 4000 μg., the 17-ethyl substituted compound similarly inactive, but 17α-ethynyl-19-norandrostane-3β,17β-diol was active at 1000 μg. The introduction of the 17α-ethyl group in 19-nor-17β-hydroxyandrostan-3-one doubled the potency whereas no change in activity occurred with the 17α-ethynyl substitution.

DISCUSSION

The method employed in this report is essentially the same as that adopted by the Cancer Chemotherapy National Service Center Endocrine Panel and is one which has been employed by one of the authors for many years. The method used by Edgren *et al.* (15) by coincidence is essentially the same as these methods as to age of mice, number of injections, and volume of oil, except that these workers used a single solution for the stimulating estrogen and the inhibitor.

We have reported previously in a preliminary communication (22) that 19-nor-17β-hydroxyandrostan-3-one was 8 to 10 times more active than the corresponding Δ^4-3-keto compound. This could not be confirmed by Edgren *et al.* (15), who judged the dihydro compound to be at most as

TABLE 6. INFLUENCE OF 17α-ETHYNYL SUBSTITUENT ON ANTI-ESTROGENIC ACTIVITY (INJECTION)

Steroid	No. of mice on steroid	No. of mice on 17α-ethynyl steroid	Steroid (A)	17α-ethynyl steroid (B)	17α-ethynyl enhancing ratio A/B
Testosterone	99	54	500	500	1.0
19-Nortestosterone	135	180	500	16	31
19-Norandrostane-3β,17β-diol	63	63	4000 Negative	1000 Negative	> 4
19-Nor-17β-Hydroxyandrostan-3-one	126	90	500	500	1.0

active as the parent compound. With additional animals added to our series, the two compounds have been found to have equal activity. Only in one instance was an increase in anti-estrogenic potency found after 5α-reduction; that is, the reduction of testosterone. Loss of the C-19 group did not consistently increase the anti-estrogenic potency. A dramatic increase was observed only for 17α-ethynyltestosterone and 17α-ethyltestosterone. Alkyl substituents, that is, methyl, ethyl, or ethynyl, at carbon 17 increased the anti-estrogenic potency in all examples studied with only one exception, 17β-hydroxyandrostan-3-one, and in 13 instances enhancement ratios of more than one to as high as 62 were found.

Of interest is the fact that 2α-methyl-17β-hydroxyandrostan-3-one is both a highly active anti-estrogen and has shown remarkably favorable

TABLE 7. COMPARATIVE RELATIVE ANTI-ESTROGEN POTENCY OF VARIOUS STEROIDS REPORTED BY EDGREN *et al.* AND THIS REPORT

Steroid	Edgren *et al.*	This report
Progesterone	1.0	1.0
19-Nortestosterone	0.4	1.0
17α-Methyl-19-Nortestosterone	8.8	15.9
17α-Ethyl-19-Nortestosterone	12.5	62.5
17α-Ethynyl-19-Nortestosterone	8.0	31.3

effects in human breast cancer (23). On the basis of this experience, it is possible that the 17α-methyl compound derivative of 2α-methyl-17β-hydroxyandrostan-3-one, which has an anti-estrogenic activity 25 times that of 2α-methyl-17β-hydroxyandrostan-3-one, may be a most effective steroid against human breast cancer. This possibility is particularly emphasized since 2α,17α-dimethyl compound was highly effective as an anti-mammary tumor agent in the rat (24).

The relative anti-estrogenic potency of compounds reported in this communication and by Edgren *et al.* (15) are reported in Table 7. All results have been referred to the same standard progesterone. The results obtained in the present study are consistently greater, by a factor of 2 to 5 times, than found by Edgren *et al.* (15). The reason for the discrepancy is not immediately apparent. It should be mentioned, however, that Edgren used a single oil solution for both the stimulating estrogen and the inhibitor, and the concentration of estrone was 0.3 μg. An oil vehicle for estrone, total dose of 0.4 μg., and the inhibitor were administered simultaneously at a different site in the present study. The inhibitor was contained in an aqueous suspension. The strains of mice used in the two laboratories were also different.

The suggested method may be described as of the titration type with an error range of about $\pm 100\%$. Thus a report of a minimum statistical response at 100 μg. may have a range from 50 μg. to 200 μg.

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THYROXINE—SERUM PROTEIN COMPLEXES: TWO-DIMENSION GEL AND PAPER ELECTROPHORESIS STUDIES

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ABSTRACT

Two dimensional (paper-paper and paper-gel) electrophoresis of human serum containing labeled thyroxine has been performed with various buffers. Thyroxine-binding prealbumin (TBPA), as seen in paper electrophoresis in ammonium carbonate buffer, appears to be identical with the fastest moving thyroxine zone (band 1) in starch gel electrophoresis in borate buffer. Albumin-bound thyroxine in ammonium carbonate or barbital buffer appears in starch gel as band 2 and a band migrating slower than band 4 the identity of which has not been established. The albumin separated on starch gel appears to be non-homogeneous with respect to its thyroxine binding. Thyroxine-binding alpha globulin (TBG) in ammonium carbonate and barbital buffer is seen in starch gel as band 4. In paper electrophoresis using barbital buffer, TBPA probably migrates ahead of albumin although a prealbumin component also appears to migrate with the alpha globulin. However, relatively high concentrations of barbital appear to inhibit binding of thyroxine by TBPA. TBG in barbital and carbonate are identical. Band 3 in starch gel has not been identified.

THYROXINE is firmly and reversibly bound to certain serum proteins but the protein species experimentally found to be responsible for binding appear to depend upon the methods used for analysis. When plasma protein preparations are obtained from Cohn fractionation all the sub-fractions of IV-4 exhibit variable amounts of thyroxine binding abilities (1). The use of paper electrophoresis with barbital buffer at pH 8.6 has revealed that an inter-alpha globulin (TBG) binds most of the plasma thyroxine at low or physiologic levels and that albumin binds significant quantities of thyroxine only at high levels (2, 3, 4). Occasionally a small amount of thyroxine bound to a pre-albumin protein has been seen in certain sera. In cerebrospinal fluid, prealbumin binds a significant amount of thyroxine (5, 6). It has recently been shown that when studies are done using a Tris(hydroxymethyl)aminomethane (Tris) and maleate buffer at pH 8.6, prealbumin consistently binds as much as $\frac{1}{3}$ of the labelled thyroxine even at low levels of thyroxine (7). It has also been shown that prealbu-

Received May 2, 1960.

min binds thyroxine when any of a variety of buffers, including sodium borate, ammonium carbonate, ammediol and phosphate (pH 8–9) are used (8, 9). Two reports have described a prealbumin as the only major thyroxine binding protein of serum when the separation is by starch gel electrophoresis using borate buffer at approximately pH 8.6 (10, 11). Other experiments have been reported which demonstrate four protein-thyroxine peaks in starch gel electrophoresis with borate buffer (9).

The present study is an attempt to identify the various thyroxine binding fractions by the use of two dimensional electrophoresis. We have used two types of experiments: in one, paper electrophoresis was used for the runs in both dimensions (paper-paper), in the second, starch gel was used for the second run. Abnormal sera, in which increases or decreases of thyroxine binding by one or more of the protein species have been observed, were also studied to assist in the identification.

METHODS

I^{131}-labeled thyroxine in 50% propylene glycol was obtained from Abbott Laboratories, Oak Ridge, Tennessee. After dilution with water it was extracted with n-butanol and repurified by an extraction procedure (12). All runs were performed with L-thyroxine which was at least 90% pure as determined by chromatography. Mixtures of serum with radioactive L-thyroxine were prepared by adding the serum to the appropriate quantity of vacuum-dried thyroxine (0.5 μg/ml. serum). The mixtures were allowed to equilibrate for at least 15 hours at 4° C, and twenty to thirty microliters of the serum-thyroxine mixture were employed in each run. Normal human serum was used in the two-dimensional runs. Serum from normal pregnant women and from patients receiving 17-methyl-19-Nor-testosterone (MNT) as therapy for breast cancer were also used.[1] All sera used was stored at −20° C. The normal serum was obtained from one individual.

One dimensional starch gel electrophoresis was performed by the method of Smithies (13) using borate buffer, pH 8.6, containing .03 moles of H_3BO_3 and 0.012 moles of sodium hydroxide per liter. The autoradiographic method described earlier (9) was used for determining the position of the radiothyroxine bands. In both types of two dimensional electrophoresis employed, viz. paper-paper and paper-gel, the serum was first run for 18 hours on a 38 mm. by 380 mm. Whatman #3MM paper strip, using the reverse-flow method (12) with either barbital buffer (pH 8.6, ionic strength 0.1) or ammonium carbonate buffer (0.1M U.S.P. grade, with pH adjusted to 8.3–8.4 by bubbling CO_2 through the solution). In the paper-paper runs, the wet strip was removed from the electrophoresis apparatus, and the section containing the radiothyroxine was placed over a rectangular hole cut in the narrow dimension of a 280 mm. by 350 mm. piece of Whatman 3MM paper. For comparison, a sample of the original serum was spotted on the sheet on one or both sides of the strip. An electrical potential was then applied across the long dimension for 18 to 24 hours. Either conventional or reverse-flow electrophoresis, and barbital or ammonium carbonate buffer, was used for the second dimension. Autoradiographs were prepared from the second dimension paper sheets, before staining with bromphenol blue. Duplicate first dimension strips were run, autoradiographed, and stained for comparison. When starch gel was employed for the second dimension run (14)

[1] We are indebted to Dr. J. Nelson, District of Columbia General Hospital, for the pregnancy serum, and to Dr. A. C. Carter, Downstate Medical Center, U. of State of N. Y., for the MNT therapy serum.

TABLE 1. SUPPORTING MEDIA, BUFFERS AND METHODS USED IN THE TWO-DIMENSIONAL ELECTROPHORESIS RUNS

No.	First dimension			Second dimension				Code Name
	Supporting medium	Buffer	Method	Supporting medium	Buffer	Method	Figure	
1	Paper	Barbital	Reverse	Paper	Amon. Carb.	Conventional	3	PBa-PAC
2	Paper	Amon. Carb.	Reverse	Paper	Barbital	Conventional	4	PA-PBaC
2	Paper	Amon. Carb.	Reverse	Paper	Barbital	Reverse Flow	—	PA-PBaR
4	Paper	Amon. Carb.	Reverse	Starch Gel	Borate	—	5	PA-S
5	Paper	Barbital	Reverse	Starch Gel	Borate	—	6	PBa-S

a strip of paper 70 mm. × 5 mm. was out from the center of the wet first dimension paper strip and inserted in the origin slit of a gel which had been cast in a 230 mm. × 80 mm. × 6 mm. tray. As a control a strip of Whatman 3MM filter paper 9 mm. × 5 mm. soaked in the original serum was placed alongside the larger strip in the origin slit. The separation on gel was carried out from 4–7 hours at a potential of 6 volts per centimeter. In each case, the approximate position of the radiothyroxine on the first dimension paper strip was determined by means of a hand-held Geiger-Mueller counter. After the second dimension run, the gel was split along its smallest dimension, making two pieces each 3 mm. × 80 mm. × approximately 100 mm. One-half was stained with amido black 10B according to the method of Smithies (13) and the other autoradiographed using "No-screen" x-ray film. Six types of two dimensional runs were done, and these are shown in Table 1. The shorthand notations introduced will be used henceforth in the text.

RESULTS

Single Dimension Studies:

After paper electrophoresis in ammonium carbonate, radioactivity was found in three bands corresponding to thyroxine-binding prealbumin (TBPA), thyroxine-binding albumin (TBA) and thyroxine-binding alpha globulin (TBG). When barbital was utilized as the buffer no band corresponding to TBPA was seen but the other two were the same. These results confirm previous findings (8).

In the single dimension starch gel runs using normal serum, four bands of radioactivity, numbered 1 to 4, were seen (Fig. 1). Band 1 corresponded to the fastest moving prealbumin (prealbumin 1 or prealbumin A in the nomenclature of Smithies (13) and Poulik and Smithies (14)); band 2 was located at the leading edge of the broad albumin zone; band 3 which was generally broad and diffuse, was in the slowest half of the albumin zone; and band 4 migrated just behind the albumin zone in a region which did not appear to correspond to any of the visible protein bands.

In single dimension starch gel runs using the sera of three pregnant women at term, most of the radioactive thyroxine was located in bands 3 and 4. In two samples of serum obtained from another woman in the 6th and 7th months of pregnancy, and compared to her pre-pregnancy serum, there was a similar increase in radioactivity in bands 3 and 4 and a decrease in band 2 (Fig. 1). Single dimension paper runs in barbital buffer showed an increase in the thyroxine-binding capacity of TBG in pregnancy sera. The

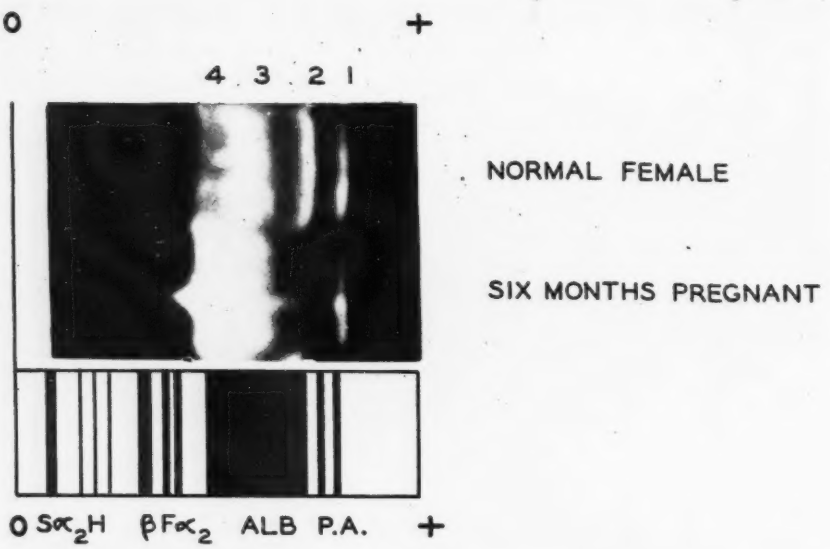

FIG. 1. Distributions of radiothyroxine in human serum after single dimension starch gel electrophoresis in borate buffer. The top two strips are radioautographs of starch gels. Top strip; serum from normal female. Middle strip; serum from the same woman in the sixth month of pregnancy. Bottom strip; diagram of distribution of serum proteins after staining with amido black. O, origin, $S\alpha_2$, slow alpha-2-globulin; β, beta-globulin, $F\alpha_2$, fast alpha-2-globulin; Alb, albumin; P.A., pre-albumin. (Nomenclature of Smithies (13).) The positive pole is indicated. The same quantity of thyroxine was added to each serum (0.5 μg./ml.).

thyroxine-binding capacity of TBPA was not determined. Starch gel electrophoresis of serum from women with breast carcinoma showed increased thyroxine in bands 3 and 4 and a decrease in band 1. After treatment with MNT there was a marked decrease in thyroxine binding in bands 3 and 4 to below normal levels (Fig. 2). Paper electrophoresis studies with barbital or ammonium carbonate buffer demonstrated a decrease in thyroxine-binding capacity of TBG in these sera as a result of MNT treatment (15). The thyroxine binding capacity of TBPA was not determined.

Two-Dimension Studies:

In the PBa-PAC studies (see Table 1), only two radioactive zones, corresponding to TBA and TBG, were seen (Fig. 3). In the PA-PBaC study, three thyroxine-binding components were disclosed (Fig. 4). TBA and TBG were represented by two distinct areas having the same mobility as the control TBA and TBG in both dimensions. In the second dimension in barbital, the radiothyroxine coming from the region corresponding to pre-

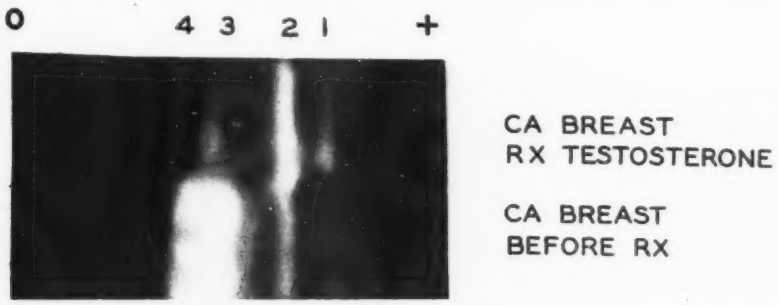

FIG. 2. Distribution of radiothyroxine in human serum after starch gel electrophoresis in borate buffer. Both strips are radioautographs of starch gels. Bottom strip; serum of patient with cancer of the breast. Top strip; the same patient while on treatment with 17-methyl-19-nor-testosterone. The same quantity of thyroxine was added to each serum (0.5 μg./ml.).

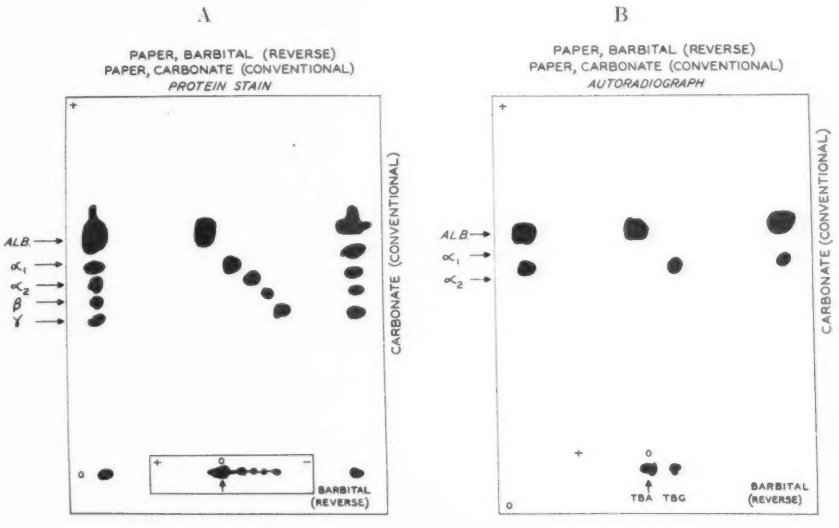

FIG. 3. Paper-paper two-dimensional run. First dimension, barbital buffer (reverse flow), second dimension, ammonium carbonate buffer (conventional). In this and subsequent figures, A is a diagram of the stained protein zones (bromphenol blue for paper and amido black for gels) and B is a diagram of the corresponding autoradiograph. α_1, alpha-1-globulin, α_2, alpha-2-globulin; β, beta globulin; γ, gamma globulin; TBA, thyroxine binding albumin; TBG, thyroxine binding alpha globulin. Samples of the original serum were run at both sides of the sheet in the second dimension in each case. At the bottom of each diagram in Figs. 3 and 4, the positions of the stained spots (A) and the radioactivity (B) in the first dimension *prior to the second dimension run* are shown.

albumin on the ammonium carbonate strip was separated into a slow component which produced a tapering streak of radioactivity starting just past the origin and ending at a point located just behind albumin, and a second component producing a small discrete spot having the mobility of prealbumin.

When the reverse-flow technique was used for the second dimension barbital run following a first dimension in ammonium carbonate, (PA-

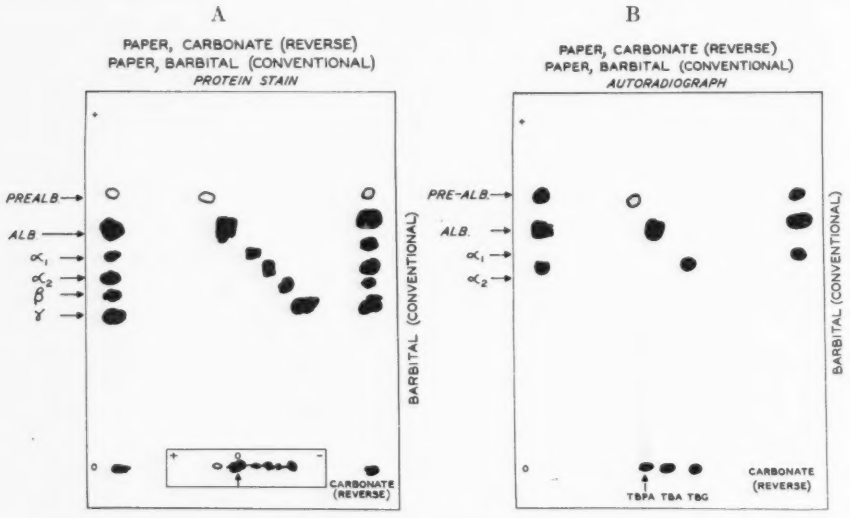

FIG. 4. Paper-paper two-dimensional run. First dimension, ammonium carbonate buffer (reverse flow); second dimension, barbital buffer (conventional). TBPA, thyroxine binding pre-albumin (See legend to Fig. 3.)

PBaR), TBPA did not separate into two components but travelled toward the anode as a single zone with the mobility of prealbumin. TBA remained fixed at the point of origin (i.e., in its place on the first-dimension strip), and TBG moved toward the cathode, in the opposite direction from prealbumin.

In the PA-S gel experiment (Fig. 5) the results were similar to those in Figure 4. The radiothyroxine migrating from the prealbumin region of the paper strip was separated into two components: a slower one which streaked from the origin, and a faster one which had the mobility of prealbumin and which corresponded in position to band 1 of the control sample. Radiothyroxine originating from the albumin zone of the paper strip was also separated into two components. The faster of these corresponded in mobility to Band 2 at the leading edge of albumin in the control sample. The other fraction travelled much more slowly and was located behind

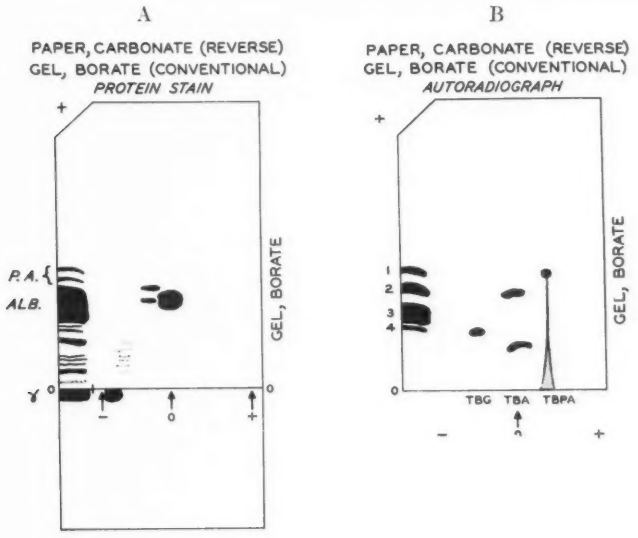

FIG. 5. Paper-gel two-dimensional run. First dimension, paper, carbonate buffer, (reverse flow). Second dimension, gel, borate buffer. (See legend to Fig. 3.)

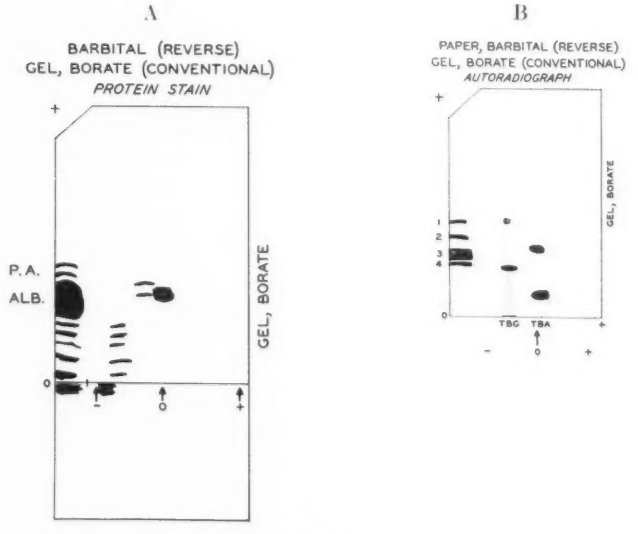

FIG. 6. Paper-gel two-dimensional run. First dimension, paper, barbital buffer (reverse flow). Second dimension, gel, borate buffer. (See legend to Fig. 3.)

Band 4 of the control in a region which did not correspond to any stained protein or any region of thyroxine-binding in the control. The TBG of the paper strip had the same mobility as Band 4 of the control. No radiothyroxine was found in the second dimension corresponding to Band 3 of the control.

When the paper-gel two-dimension experiment was performed with barbital as the first dimension, the results were somewhat different (Fig. 6). The radioactivity from the albumin region on the paper moved as two zones, one with a mobility intermediate between those of Bands 2 and 3 and the other slower Band 4. Radiothyroxine arising from the TBG region of the paper strip produced a streak extending from the origin to Band 1 of the control; however, there was a spot corresponding to Band 1 and a much stronger band of radioactivity in a position corresponding to Band 4.

DISCUSSION

The purpose of the present study is to inter-relate the thyroxine-binding proteins separated by starch gel electrophoresis and those separated by paper electrophoresis using both barbital and ammonium carbonate buffers.

Band 1—TBPA

The paper-gel two-dimensional experiment in which ammonium carbonate was the buffer in the paper run (PA-S), clearly shows that at least some of the labeled thyroxine associated with TBPA migrates in the starch gel to the position of band 1 (Fig. 5). The streaking from the origin could be due to dissociation of thyroxine from TBPA. The observation that the trailing thyroxine becomes less as electrophoresis progresses favors this view, since a decrease in quantity of thyroxine bound to the protein would tend to favor binding to the protein as opposed to the gel. In the absence of protein, thyroxine does not migrate into the gel.

In the paper-paper, two dimension experiments in which ammonium carbonate was the first dimension, the thyroxine associated with TBPA also moved faster than albumin in barbital buffer. When the second dimension was run by the conventional technique (PA-PBaC) (Fig. 4), however, trailing similar to that found in the paper-gel experiment was observed. This could be due to dissociation of thyroxine from TBPA or the presence of two TBPA components, only one of which exhibited trailing. The amount of trailing in the paper-paper runs, however, was considerably more than in the paper-gel runs. An interference by barbital in the interaction between thyroxine and TBPA has been postulated as the cause for the absence of a thyroxine-TBPA band in single dimension electrophoresis in barbital (7, 16). This view is supported by the observation that a thyroxine-TBPA complex may be found in barbital electrophoresis of cerebrospinal fluid,

where there is a relative increase in TBPA (5), assuming that it is the same protein present in blood. Furthermore, some thyroxine migrates with TBPA when a low concentration of barbital is employed (17). The absence of TBPA in the paper-paper experiment in which barbital was the first dimension could be explained by the migration of TBPA ahead of albumin without any thyroxine bound to it. In the paper-gel experiments with barbital as the first dimension, however, the findings were different since a small thyroxine zone with the mobility of prealbumin moved out of the TBG area, again with considerable trailing. It is possible that this component is related to the prealbumin of Schultze (18) which was studied by Tata (19). This protein binds thyroxine, appears to migrate with TBG in barbital buffer in the presence of whole serum, and has been found to move with prealbumin in starch gel (11). The suggestion that this prealbumin is different from TBPA (16, 20) is supported by the present experiments (cf. Fig. 3).

It is of interest that no band 1 was obtained from the albumin zone in barbital-gel experiments, since the presence of TBPA in this zone is sometimes suggested by a shoulder on its leading edge (9). This discrepancy may be due to the fact that this "TBPA" zone, when present, is quantitatively very small.

The different findings obtained by Rich and Bearn (10) and Allison (11) in single dimension gel experiments, in which they found most of the thyroxine, at low concentrations, in the location of Band 1, has not been explained by the present experiments. Presumably they are the result of differences in technique. The proteins separated in starch gel are in such narrow bands that cutting the gel and determining radioactivity in the sections, as these authors did, could blur sharply localized areas of radioactivity. It would be of interest to determine by two dimensional experiments whether or not the fast band in their experiments contains TBG, as they proposed.

Bands 2 and 3—Albumin

Thyroxine associated with albumin in both barbital and ammonium carbonate buffers migrates to or near the position of band 2 in starch gel. Band 2 occupies a discrete area in the leading edge of the albumin zone both on single and two dimension starch gel runs. It is uncertain whether this indicates the presence of a specific protein different from albumin which binds thyroxine, or whether the thyroxine-albumin complex might have a different mobility in starch gel than does free uncomplexed albumin.

In addition to band 2, there is a thyroxine-binding band slower than band 4 arising from the albumin zone of the first dimension. There was no corresponding stained protein zone, and there is no information at present to explain its origin.

Band 3 is not seen in starch gel electrophoresis when a separation is first performed in paper using either barbital or ammonium carbonate buffer. No explanation for this phenomenon is apparent. Recent work, to be reported later (21), has shown that the material localized in this band binds nearly all the thyroxine when extremely large amounts (~60 μg/ml.) of thyroxine are added to serum. It is probable, therefore, that this represents an albumin fraction. The apparent changes in band 3 in abnormal sera are unexplained.

Band 4—TBG

Band 4 in starch gel appears to correspond to TBG in both barbital and ammonium carbonate paper electrophoresis runs (Fig. 5, 6). The detection of a second smaller component in TBG separated by barbital buffer has been discussed above. Further evidence that band 4 and TBG are equivalent is derived from the experiments using the serum from pregnant women and from individuals receiving MNT (Figs. 1, 2). The increase in the thyroxine-binding capacity of TBG in pregnancy (16, 22, 23) and the decrease in TBG capacity produced by MNT (15) are accompanied by an increase and a decrease, respectively, in thyroxine associated with band 4 at relatively low thyroxine concentration. Furthermore, the equivalence of TBG in the paper-paper, two dimension experiments is consistent with the observation that thyroxine-binding capacity of TBG is the same when measured in either buffer (8, 9). The difference between TBG in barbital and ammonium carbonate as revealed by the paper-gel runs, however, requires further investigation in this regard. It may be that any differences are quantitatively within the limits of error of the technique.

The small amount of thyroxine in band 1 (not visible in the illustration [Fig. 2]) in the serum of a patient with carcinoma of the breast is of interest. This may be analogous to the decreased levels of TBPA seen in chronically ill individuals as previously reported by Ingbar (16).

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STUDIES ON THE STATE OF INSULIN IN BLOOD: DISSOCIATION OF PURIFIED HUMAN BLOOD INSULIN COMPLEX(ES) BY INCUBATION WITH ADIPOSE TISSUE EXTRACTS *IN VITRO*[1]

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ABSTRACT

Blood insulin complexes examined for insulin activity *in vitro* by the rat diaphragm assay under specified conditions did not exhibit full insulin activity. Incubation of the blood insulin complexes with crude and purified human and rat adipose tissue extracts resulted in an increase of insulin activity in the resin eluates. Extracts from other tissues, such as human liver, kidney and muscle and from rat diaphragm, did not produce similar results. It is suggested that adipose tissue extracts may contain a system which produces either free insulin from the blood insulin complexes or other forms of insulin which could be utilized by the rat diaphragm.

IT HAS been reported that basic protein-insulin complexes extracted from blood or serum with the use of cationic exchange resin (Na^+ cycle) (1–3) did not exhibit full insulin activity when examined by the rat diaphragm tissue assay (4) under specified conditions (5). Removal of the basic protein carrier from the complex by centrifugation at pH 10 resulted in an increase of insulin-like activity. Identical preparations, however, exhibited insulin-like activity when examined by the rat adipose tissue assay (6) without prior dissociation of the insulin from its complexed form.

These observations suggested a difference in the mechanism of utilization of the blood insulin complexes by these tissues *in vitro*. The supposition that the rat adipose tissue could utilize insulin in its complexed form through a mechanism present in this tissue resulted in the undertaking of the *in vitro* studies presented in this communication. The results indicate that such a mechanism is present in extracts obtained from human and rat adipose tissue. Blood insulin complexes examined for insulin activity by the

Received May 20, 1960.

[1] This work was supported by grants from the National Foundation and Eli Lilly and Company. The blood serving as a source for the extraction of insulin and basic protein-insulin complexes was collected and processed under a grant (H-2929) from the National Heart Institute.

[2] Recipient of Special Research Fellowship, N.I.H., Division of Arthritis and Metabolic Diseases.

rat diaphragm tissue assay in the presence of such extract exhibited insulin activity, whereas without the presence of such extracts identical insulin complexes were either devoid of insulin activity or exhibited low activity.

Extracts from other tissues such as rat diaphragm and human liver, kidney and muscle did not contribute to the release of insulin activity under identical conditions.

MATERIALS AND METHODS

Extraction of Blood Insulin Complex(es): The methods for the extraction of the insulin complex(es) from blood with the use of cationic exchange resin (Na^+ cycle) have been described elsewhere (3). In these studies the following five preparations from normal subjects have been employed:

Lot 016 represents the extract from four liters of blood collected through cationic exchange resin (Na^+ cycle). The resin columns were washed with 0.15 M NaCl and eluted with 0.1N H_2SO_4 (pH 2.0). The combined resin eluates were dialyzed against 0.02 M NaCl and lyophilized. The dried material was dissolved in Gey and Gey bicarbonate buffer, dialyzed against ten volumes of the same buffer, and assayed for insulin activity.

Lo's 080, 081, 082 and 101 were eluted from the resin columns (by ammonium hydroxide solutions) (3) following blood collection. The eluates were dialyzed against 0.02 M NaCl and lyophilized. The dried material was dissolved in Gey and Gey bicarbonate buffer, dialyzed against the same buffer, and assayed for insulin activity. Lots 080, 081 and 082 represent the extract from 2–3 liters of plasma each, and lot 101 represents that from about 20 liters of plasma.

Preparation of Tissue Extracts: Rat Adipose Tissue Extract (*RATE*) was obtained by homogenizing epididymal adipose tissue of male rats (weighing 80 to 120 gm.) with Gey and Gey bicarbonate buffer. The epididymal adipose tissue prior to extraction was kept frozen at −20° C. Two to five grams of the frozen tissue was homogenized in a Teflon tissue grinder with 10 to 20 ml. of cold Gey and Gey bicarbonate buffer at 2° C. The homogenate was centrifuged at 3,000 r.p.m. at 2° C. for 30 minutes, and the fat-free fluid portion of the homogenate was collected. When necessary, the centrifugation was repeated in order to remove the fat contaminating the tissue extract. The adipose tissue extract was stored at −20° C. *Human and Bovine* adipose tissue extracts (mesenteric fat) were obtained with Gey and Gey bicarbonate buffer with a method similar to that employed for the preparation of the rat adipose tissue extract.

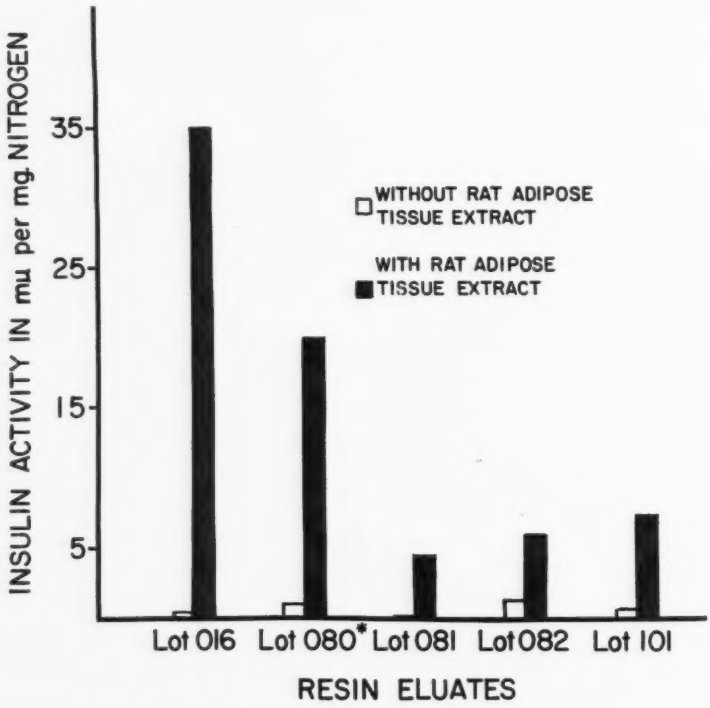
Extracts from human liver, kidney and muscle and rat diaphragm were also prepared with Gey and Gey bicarbonate buffer. The tissues were stored at −20° C. prior to homogenization. Samples from human tissues were collected from nondiabetics post mortem. The tissues were homogenized from the frozen state in a Teflon tissue grinder as described above. The tissue extracts were stored at −20° C.

Partially purified human, bovine or rat adipose tissue extracts were obtained by cold ethanol precipitation. 10 to 20 ml. of the crude extract were brought to 60% ethanol concentration at −5° C under continuous stirring with the use of 95% ethanol at pH 7.2 ± 0.2. The mixture was stored at −5° C overnight, and the precipitate was removed by centrifugation at −5° C at 3,000 r.p.m. for 30 minutes. The supernatant fluid was diluted with cold distilled water in order to bring the ethanol concentration to 4–6%. The mixture was then lyophilized from the frozen state. The dried material was dissolved with sufficient cold distilled water to bring the volume to that of the original volume of the crude extract employed for the purification. The purified extract was stored at −20° C.

Assay of Insulin Activity: Insulin-like activity was determined by the rat diaphragm tissue assay (4). This method is based on the increment of glucose uptake by this tissue in the presence of insulin.

RESULTS

Lyophilized resin eluates were dissolved in Gey and Gey bicarbonate buffer (15 mg. of resin eluate per ml. buffer) and dialyzed against ten volumes of the same buffer. Part of the solution was assayed for insulin



* Incubated with human adipose tissue extract.

FIG. 1. Bioassay of blood insulin complex(es) before and after incubation with rat adipose tissue extracts.

activity by the rat diaphragm tissue assay directly and without the presence of tissue extract. On the same day an identical portion of the resin eluate was assayed for insulin activity in the presence of the tissue extract (0.5–1.0 ml. of the extract was added to 10 ml. of incubating medium).

Figure 1 shows that incubation of the various eluates with rat adipose tissue extract resulted in a many-fold increase of insulin activity in the eluate as compared with the control eluate, which was assayed without in-

TABLE 1. INSULIN ACTIVITY OF BLOOD RESIN ELUATES BEFORE AND AFTER INCUBATION WITH HUMAN ADIPOSE TISSUE (HAT) AND RAT ADIPOSE TISSUE (RAT) EXTRACTS

Lot No.	Material	Total glucose uptake in μg. per 10 mg. dry diaphragm ± S.E.	
016*	Lot 016 without RAT extract	90 ± 8‡	62 ± 8
	Lot 016 with RAT extract (55 μg. nitrogen)†	132 ± 6	228 ± 22
080*	Lot 080 without HAT extract	92 ± 14	60 ± 9
	Lot 080 with HAT extract (36 μg. nitrogen)†	222 ± 6	224 ± 26
081*	Lot 081 without RAT extract	20 ± 11	
	Lot 081 with RAT extract (36 μg. nitrogen)†	220 ± 15	
082*	Lot 082 without RAT extract	102 ± 11	
	Lot 082 with RAT extract (36 μg. nitrogen)†	166 ± 0	
101*	Lot 101 without RAT extract	36 ± 10	86 ± 9
	Lot 101 with RAT extract (18 μg. nitrogen)†	178 ± 10	288 ± 9
	RAT extract alone (n-6)	−4 ± 9	
	HAT extract alone (n-6)	0 ± 2	
	Bicarbonate buffer alone (n-12)	208 ± 4	

* Dialyzed overnight at 2° C against 20 volumes of Gey and Gey bicarbonate buffer.
† Nitrogen content of the extract per ml. incubating medium used in each determination.
‡ All determinations were made in triplicate and calculated as net glucose uptake above control (buffer alone).

cubation with the extract. The tissue extract alone (0.5–1.0 ml.) added to 10 ml. of Gey and Gey bicarbonate buffer had no insulin activity.

Table 1 summarizes the results of these experiments, including the data for the control tissue extract and the control eluates.

Figure 2 shows the effect of the concentration of the rat adipose tissue extract on the increase of the insulin activity in the resin eluate. A crude tissue extract with a nitrogen content of 18 μg. per ml. of incubating medium was adequate for the increase of the insulin activity in the eluate. Lower concentrations of rat adipose tissue extract incubated with insulin complex(es) under the same conditions released lower amounts of insulin from

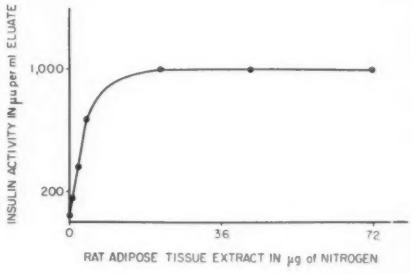


FIG. 2. Effect of concentration of rat adipose tissue extract on the release of insulin activity from blood insulin complex(es).

TABLE 2. THE EFFECT OF DILUTION ON THE ACTIVITY OF RAT ADIPOSE TISSUE EXTRACT

Lot No.	RAT extract μg. nitrogen per ml. incubating medium	Total glucose uptake in μg./10 mg. dry diaphragm+S.E.	
		Exp. 1	*Exp. 2*
101*	0	86 ± 9†	
	2.1	148 ± 13	
	21	184 ± 8	
	36		180 ± 5
	216	288 ± 9	266 ± 3
	432		236 ± 1
	720		248 ± 4

* Dialyzed overnight at 2° C against 200 volumes of Gey and Gey bicarbonate buffer.
† All determinations were made in triplicate and calculated as net glucose uptake above control (buffer alone).

the eluate, and in the absence of the tissue extract the insulin activity was not significant (Table 2).

Effect of pre-incubation time: Rat adipose tissue extracts were pre-incubated with resin eluates for 5 to 60 minutes at 37° C before the incubation with the rat diaphragm tissue required by the assay. Figure 3 shows that pre-incubation of the eluate with the tissue extract for 30 minutes results in optimal release of the insulin activity in the resin eluate.

Separation of the Activity from Rat Adipose Tissue Extract by Paper Electrophoresis

A portion of a purified extract (60% ethanol supernatant) obtained from rat adipose tissue was applied on paper strips (S and S 470) and subjected to electrophoresis for 16 hours in phosphate buffer, pH 7.4 at 35 ma. at 2° C. A paper strip carrying human plasma was run in the same machine simultaneously. The paper strip carrying the plasma control was stained for proteins, and the paper strips carrying the adipose tissue estract (duplicate experiment) were cut into sections corresponding to the γ-globulin, β-globulin, α_2-globulin and α_1-globulin/albumin zone. The extract from each paper cut was incubated with the resin eluate (lot 081). As is shown in Figure 4, the extract from the α_1-globulin/albumin zone dem-

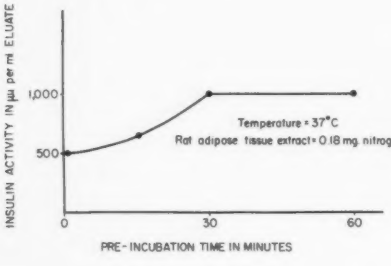
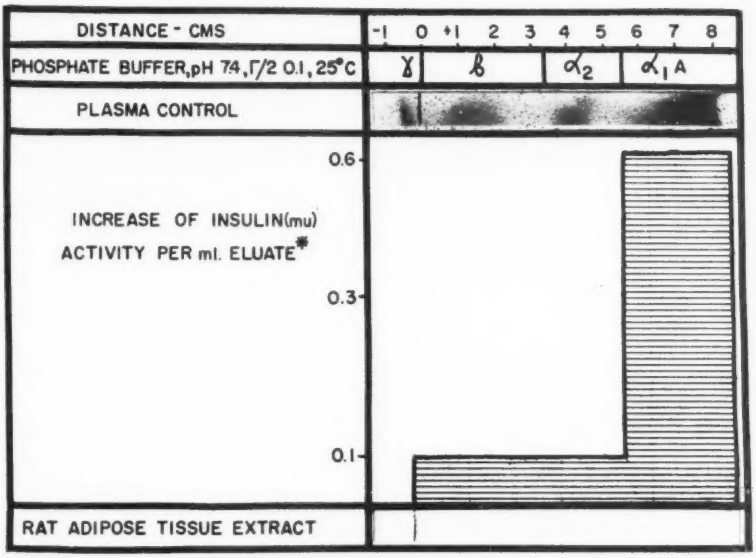


FIG. 3. Effect of pre-incubation time on the release of insulin activity from blood insulin complex(es) in the presence of rat adipose tissue extracts.



* The paper strip carrying the adipose tissue extract was cut in sections. The extract from each paper cut was incubated with the resin eluate (lot 081). The extract of the α_1-gloculin/A zone caused an increase of insulin activity in the resin eluate. The insulin activity of an identical portion of lot 081 without the presence of the adipose tissue extract or extracts from the paper cuts was only 0.025 mu/ml.

FIG. 4. Separation of the active component(s) in the rat tissue extract by paper electrophoresis.

onstrated a significant increase of insulin activity in the resin eluate as compared with the other sections of the paper strip. The control (resin eluate alone) showed only 0.025 mu of insulin activity per ml. eluate.

DISCUSSION

Previous reports suggested that insulin in blood circulates in a complex form with other substances and, more specifically, with proteins having a large basic net charge (1–3). These basic protein-insulin complexes could be adsorbed from blood or serum on cationic exchange resin (Na^+ cycle) at physiological pH. Commercial crystalline insulin dissolved in albumin, human serum, or injected intravenously into nondiabetic subjects failed to adsorb on this resin under identical contitions.

The transport of insulin in blood by other proteins was suggested to serve as a means for the regulation of the insulin activity in blood through an equilibrium between "bound" and "free" insulin. It was further suggested that the "free" insulin in blood could represent the active portion of

this hormone available to the tissue (3). Evidence that insulin in its bound form is biologically inactive was obtained by *in vitro* assays under specified conditions (3, 5). Dissociation of the insulin complexes by centrifugation at pH 10 resulted in the demonstration of insulin-like activity in these preparations (5).

Dissociation of purified blood insulin complexes can be also achieved, as described in the present report, by incubating the insulin complexes with adipose tissue extracts of various species. The use of adipose tissue extracts for the dissociation of blood insulin complexes resulted from the observation that the intact rat adipose tissue could utilize insulin in its complex form. This observation suggested that this tissue may contain a mechanism for the utilization of bound insulin. Both crude and purified adipose tissue extracts demonstrated the presence of such a mechanism.

The increase of insulin activity in the blood insulin complexes depends both upon the concentration of the adipose tissue extract and upon the time of incubation with these complexes. Control experiments demonstrated that the adipose tissue extracts alone were devoid of insulin activity. The activity of commercial crystalline insulin was not affected by the presence of adipose tissue extracts.

Other tissue extracts obtained from human liver, kidney, muscle and rat diaphragm did not have a similar effect on the insulin activity of the blood insulin complexes.

The nature of the mechanism of the increase of insulin activity in the blood insulin complexes in the presence of adipose tissue extracts is not as yet known. It is possible that the utilization of the blood insulin complexes by the rat diaphragm, following incubation with the adipose tissue extracts, results from the intervention of enzymes present in the adipose tissue extract. Such enzymes may affect the rate of dissociation of the insulin from its complex form, the free insulin exerting its activity on the rat diaphragm. Other mechanisms of action, not understood presently, may also be responsible for the effect of the adipose tissue extracts on the blood insulin complexes.

Acknowledgments

We wish to express our appreciation to Mrs. Elizabeth Goolsby and Mr. Anatolii Echardt for their helpful assistance throughout this work.

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ANTI-ESTROGEN ASSAY OF NEUTRAL STEROIDS ADMINISTERED BY GAVAGE[1,2]

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ABSTRACT

A three day test for the evaluation of oral anti-estrogenic activity is described using 20–23-day-old female Swiss albino mice which are stimulated with subcutaneously administered estrone. The test compound is administered simultaneously by gavage. The end-point is the degree of inhibition of estrogen produced uterine growth. The relative anti-estrogenic potencies of 25 steroids are reported on the basis of the minimum weight of compound, administered by gavage, needed to produce a statistically significant inhibition of the uterine stimulation produced by 0.4 μg. of estrone. Correlations of structure and activity may be summarized as follows: reduction of the Δ^4-3-keto group in ring A to the 5α-3-keto caused either a decrease in activity or no net change, loss of carbon 19, with few exceptions, caused increased activity, and the introduction of 17α-alkyl groups resulted in increased anti-estrogenic activity in about one-half the cases studied.

The anti-estrogenic activity of steroids was, with only one or possibly two exceptions, greater by subcutaneous injection than by gavage. Other comparative figures are given for relative potencies by subcutaneous injection and gavage.

IN A previous paper a test for anti-estrogenic activity was suggested in which the inhibitor was administered subcutaneously and certain correlations between steroid structure and biological activity were presented (1). The present communication describes a parallel study where the inhibitors were administered by gavage. Specifically, this report deals with the relative anti-estrogenic potency of steroids administered by gavage, correlation of structure and relative potency by this route, and finally a comparative study of selected steroids administered by both subcutaneous injection and by gavage.

METHODS AND MATERIALS

Twenty- to twenty-two-day-old Swiss albino mice were subcutaneously injected with estrone once daily for three days. The total dose of 0.4 μg. estrone was contained in 0.3 ml. of sesame oil and 0.1 ml. was injected each day. The total dose of the test compound

Received June 1, 1960.

[1] Supported in part by grant No. CY-2193 from the U. S. Public Health Service.

[2] This is Steroids CLII in the series published from the Syntex Research Laboratories.

was suspended in 0.6 ml. of an aqueous medium, 0.2 ml. being administered daily by gavage for three days. The aqueous medium consisted of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethylcellulose (0.5%), and benzyl alcohol (0.9%). One day after the last injections the mice were sacrificed and the uterine and body weights were determined.

The assays were run in lots of 132 mice which were divided into 14 groups in a manner similar to that described previously (1). One group of 12 mice served as sesame oil injected control animals, another group of 12 mice received a total dose of 0.4 μg. estrone. The remaining 12 groups of 9 mice each received the test compounds. Usually a single compound was studied at three or more concentrations in any specific assay. Finally, to arrive at the relative activity of a given compound, all the data for a specific compound at a specific dose were combined. To determine the minimum dose to produce inhibition, a response was chosen which when calculated statistically gave a value for P of less than 0.02.

RESULTS

The relative anti-estrogenic activity of various steroids administered by gavage is presented in Table 1. The compounds are listed in the order of decreased potency on the basis of the minimum quantity necessary to produce a statistically significant inhibition. Also tabulated is the maximum inhibition attained at any dose studied. This was calculated from the mean uterine weight of the mice treated with 0.4 μg. of estrone. For example, if the uterine weight was 50 mg. for 0.4 μg. of estrone, and the mean uterine weight for a hypothetical group treated both with 0.4 μg. of estrone and an effective inhibitor was 25 mg., the inhibition was recorded as 50%. This is low compared to a calculation which would correct for the uterine weights of the sesame oil treated mice. In the latter case the uterus would be about 10 mg. and the stimulation would account for 40 mg. of tissue. The net stimulation for estrone plus inhibitor would then be 15 mg. and the per cent inhibition would be 63%.

TABLE 1. RELATIVE ANTI-ESTROGENIC ACTIVITY OF STEROIDS ADMINISTERED BY GAVAGE (0.4 μg. Estrone Injected Subcutaneously)

Steroid	Total No. of mice	Dosage range studied (μg.)	Minimum dose to produce inhibition (μg.)	Maximum inhibition %
17α-Methyl-19-Nortestosterone	265	5– 500	40	44
17α-Ethyl-19-Nortestosterone	227	40–10,000	40	43
17α-Ethynyl-19-Nortestosterone	238	2– 1,000	32	40
17α-Methyltestosterone	270	4– 2,000	250	36
17α-Ethynyl-19-Norandrostane-3β,17β-Diol	105	100– 2,000	500	34
Androstane-3β,17β-Diol	88	50–10,000	1,000	36
19-Nortestosterone	118	10– 4,000	1,000	17
17α-Methyl-19-Nor-17β-Hydroxyandrostan-3-one	117	100– 2,000	1,000	24
Desoxycorticosterone	118	10– 4,000	2,000	30
17α-Methyl-17β-Hydroxyandrostan-3-one	209	1– 4,000	2,000	22
17α-Methyl-19-Norandrostane-3β,17β-Diol	106	10–10,000	4,000	40
19-Nor-17β-Hydroxyandrostan-3-one	159	100–10,000	4,000	36
Testosterone	309	10– 4,000	4,000	10
17α-Ethynyl-19-Nor-17β-Hydroxyandrostan-3-one	30	1,000–10,000	4,000	12
19-Norandrostane-3β,17β-Diol	27	1–10,000	4,000	20
17α-Vinyl-19-Norandrostane-3β,17β-Diol	27	1,000–10,000	4,000	11
17β-Hydroxyandrostan-3-one	159	5– 4,000	4,000	24
17α-Ethynyltestosterone	103	50– 4,000	4,000	20
17α-Methyl-Δ^5-Androstene-3β,17β-Diol	288	1– 4,000	4,000 (Negative)	
17α-Ethyl-19-Norandrostane-3β,17β-Diol	24	1,000–10,000	10,000 (Negative)	
19-Nor-Δ^5-Androstene-3,17-Dione	72	10– 1,000	1,000 (Negative)	
17α-Ethyltestosterone	216	1– 4,000	4,000 (Negative)	
17α-Vinyl-19-Nor-17β-Hydroxyandrostan-3-one	27	1,000–10,000	10,000 (Negative)	
Progesterone	120	50– 1,000	1,000 (Negative)	

TABLE 2. INFLUENCE OF LOSS OF C-19 (FORMATION OF 19-NOR DERIVATIVE) ON ANTI-ESTROGENIC ACTIVITY (GAVAGE)

Steroid	No. of mice on steroid	No. of mice on 19-nor steroid	Lowest total dose (μg.) producing inhibition		19-nor. enhancing ratio = A/B
			Steroid (A)	19-nor steroid (B)	
Testosterone	309	118	4000	1000	4
17α-Methyltestosterone	270	265	250	40	6.5
17α-Ethyltestosterone	216	227	4000	40	>100
			(Negative)		
17α-Ethynyltestosterone	103	238	4000	100	40
Δ⁴-Androstene-3,17-Dione	36	72	500	1000	—
			(Negative)	(Negative)	
17β-Hydroxyandrostan-3-one	101	159	4000	4000	1.0
Androstane-3β-17β-Diol	88	27	1000	4000	0.25
17α-Methyl-17β-Hydroxy-androstan-3-one	209	117	2000	1000	2.0

The removal of carbon 19 produced increased activity in five instances, no change in one, and a decreased activity in one case (Table 2). The relative potency of Δ⁴-androstene-3,17-dione and the 19-nor derivative could not be determined with certainty since no significant inhibitions could be determined. Most striking was the enhancement of anti-estrogenic action of >100 and 40 times for the changes brought about by the removal of carbon 19 from 17α-ethyltestosterone and 17α-ethynyltestosterone, respectively.

Reduction of the Δ⁴-double bond to the 5α dihydro derivative (Table 3) did not result in any significant increase in anti-estrogenic activity, but rather a severe reduction of activity in one instance. The minimum dose showing a statistically significant decrease for 17-methyltestosterone was increased from 250 μg. to 2000 μg. on saturation of the Δ⁴-double bond.

Great variations in anti-estrogenic activity resulted from the replacement of the 17α-hydrogen by a 17α-methyl group (Table 4). Anti-estrogenicity was sharply decreased by this change for Δ⁵-androstene-3β,17β-diol and 19-norandrostane-3β,17β-diol, where enhancing ratios of <0.25 and 0.1, respectively, were found. True enhancement was found for testo-

TABLE 3. INFLUENCE OF REDUCTION OF Δ⁴ GROUP TO 5α CONFIGURATION ON ANTI-ESTROGENIC ACTIVITY (GAVAGE)

Steroid	No. of mice on steroid	No. of mice on 5α-steroid	Lowest total dose producing inhibition (μg.)		5α-reducing enhancing ratio = A/B
			Steroid (A)	5α-steroid (B)	
17α-Ethyltestosterone	216	45	10,000	Negative at 1,000	—
			(Negative)		
17α-Methyltestosterone	270	209	250	2,000	0.13
Testosterone	309	101	4,000	4,000	1.0
19-Nortestosterone	118	63	1,000	Negative at 1,000	<1

TABLE 4. INFLUENCE OF 17α-METHYL SUBSTITUENT ON ANTI-ESTROGENIC ACTIVITY (GAVAGE)

Steroid	No. of mice on steroid	No. of mice on 17α-Methyl steroid	Lowest total dose producing inhibition (μg.)		17α-methyl enhancing ratio A/B
			Steroid (A)	17α-methyl steroid (B)	
Testosterone	309	270	4,000	250	16
19-Nortestosterone	118	180	1,000	40	25
19-Nor-17β-Hydroxy-androstan-3-one	159	72	10,000 (Negative)	10,000 (Negative)	—
Δ^5-Androstene-3β,17β-Diol	88	288	1,000	4,000 (Negative)	< 0.25
17β-Hydroxyandrostan-3-one	101	209	4,000	2,000	2
19-Norandrostane-3β,17β-Diol	27	106	1,000	10,000	0.1
17β-Diol					

sterone (16 times), 19-nortestosternoe (25 times), and 17β-hydroxyandrostan 3-one (2 times).

An enhancement ratio of 25 times was observed for the 17α-ethyl modification of 19-nortestosterone but actual decreases in activity were found for the two other compounds (Table 5). The enhancement ratio was less than 1 for testosterone and that for 19-norandrostane-3β,17β-diol less than 0.1. No decreases in enhancement ratio were found when the 17α-ethynyl group was substituted for the 17α-hydrogen but the activity of two compounds remained unchanged (Table 6). These steroids were testosterone and 17β-hydroxyandrostan-3-one. 19-Nortestosterone was changed from a compound active at 1000 μg. to one active at 32 μg. by the introduction of the ethynyl group. Of particular interest was the fact that the anti-estrogenic activity of 19-norandrostane-3β,17β-diol could be enhanced 2 times with a 17α-ethynyl group in contrast to the severe loss of activity on the addition of either the 17α-ethyl or 17α-methyl groups where enhancement ratios of <0.1 and 0.1 were found, respectively.

TABLE 5. INFLUENCE OF 17α-ETHYL SUBSTITUENT ON ANTI-ESTROGENIC ACTIVITY (GAVAGE)

Steroid	No. of mice on steroid	No. of mice on 17α-ethyl steroid	Lowest total dose producing inhibition (μg.)		17α-ethyl enhancing ratio =A/B
			Steroid (A)	17α-ethyl steroid (B)	
Testosterone	309	216	4,000	4,000 (Negative)	< 1.0
19-Nortestosterone	118	227	1,000	40	25
19-Norandrostane-3β,17β-Diol	27	24	1,000	10,000 (Negative)	< 0.1

TABLE 6. INFLUENCE OF 17α-ETHYNYL SUBSTITUENT ON ANTI-ESTROGENIC ACTIVITY (GAVAGE)

Steroid	No. of mice on steroid	No. of mice on 17α-ethynyl steroid	Lowest total dose producing inhibition (μg.)		17α-ethynyl enhancing ratio = A/B
			Steroid (A)	17α-ethynyl steroid (B)	
Testosterone	309	103	4000	4000	1.0
19-Nortestosterone	118	238	1000	32	31
19-Norandrostane-3β,17β-Diol	27	105	1000	500	2.0
17β-Hydroxyandrostan-3-one	101	30	4000	4000	1.0

DISCUSSION

The reduction of the Δ^1-3-keto group in ring A resulted in either no change or a loss in biological activity. No enhancement of activity was found for the four compounds studied. Enhancement of anti-estrogenic activity, however, was found for 5 of the 8 compounds studied in which loss of carbon 19 was the structural modification. This enhancement varied from a factor of 2 for 17α-methyl-17β-hydroxyandrostan-3-one to over 100 for 17α-ethyltestosterone. The introduction of 17α-alkyl groups for the 17α-hydrogen atoms enhanced the biological activity in 6 of the 13 instances. The enhancement factor from testosterone to methyltestosterone was 16, which was expected on the basis of the known high oral androgenic activity of the methyl compound compared to the low activity of the parent compound.

The anti-estrogenic activity of all steroids, with one exception, was significantly greater when administered by subcutaneous injection than by the oral route. Some of these differences are indeed striking (Table 7). For example, 17β-hydroxyandrostan-3-one was 40 times more active by injection and 17α-methyl-19-nor-17β-hydroxyandrostan-3-one was 20 times more active by the same route. One exception was the case of 17α-methyl-19-nortestosterone which produced a minimum effect at 32 μg. subcutaneously and a similar effect at 40 μg. by gavage. This difference in dosage is not significant. This may also be true for 17α-ethynyl-19-nortestosterone, where the subcutaneous dose of 16 μg. and gavage dose of 32 μg. are perhaps not significantly different. On the other hand, the relative potency of 17-ethyl-19-nortestosterone by the two routes shows a ratio of 5 for subcutaneous to gavage administration efficiency.

When enhancing ratios for certain structural modifications were compared for the subcutaneous and gavage routes, interesting and surprising differences were observed. As indicated in Table 8, the dihydro (5α) derivative of testosterone is equal to that of testosterone by gavage, but by subcutaneous injection the dihydro derivative was 5 times more active.

Excellent agreement for the two routes is seen for structural changes

TABLE 7. THE COMPARATIVE ANTI-ESTROGENIC ACTIVITY OF VARIOUS STEROIDS BY SUBCUTANEOUS INJECTION

(Data on Subcutaneous Injection (SI) from Dorfman *et al.* (1959) and by Gavage (G))

Steroid	Minimum dose to produce inhibition (μg.)		Maximum inhibition %	
	SI	G	SI	G
17α-Ethyl-19-Nortestosterone	8	40	54	43
17α-Ethynyl-19-Nortestosterone	16	32	56	40
17α-Methyl-19-Nortestosterone	32	40	46	44
17α-Methyltestosterone	32	250	35	35
17α-Methyl-19-Nor-17β-Hydroxyandrostan-3-one	50	1,000	38	24
17β-Hydroxyandrostan-3-one	100	4,000	30	24
17α-Ethyl-19-Nor-17β-Hydroxyandrostan-3-one	250		27	
Androstane-3β,17β-diol	250	1,000	22	36
Testosterone	500	4,000	36	10
19-Nor-17β-Hydroxyandrostan-3-one	500	4,000	32	36
19-Nortestosterone	500	1,000	33	17
17α-Ethynyl-19-Nor-17β-Hydroxyandrostan-3-one	500	4,000	27	12
17α-Vinyl-19-Nor-17β-Hydroxyandrostan-3-one	500	>10,000	20	
17-Ethynyltestosterone	500	> 4,000	22	
17α-Ethynyl-19-Norandrostan-3β,17β-Diol	1,000	500	14	34
19-Norandrostane-3β,17β-Diol	>4,000	4,000		20

involving loss of carbon 19 for 17α-ethynyltestosterone and 17α-ethyltestosterone. The enhancing ratios by the two routes were quite similar for these compounds (Table 9). This was not the case for the other four compounds and their corresponding 19-nor derivatives. In all other instances a significantly higher enhancing ratio was found by gavage. The ratios of the

TABLE 8. COMPARATIVE ENHANCING RATIOS AS A RESULT OF Δ^4-REDUCTION (5α) BY SUBCUTANEOUS INJECTION (SI) AND GAVAGE (G)

Steroid	Enhancing ratio	
	SI	G
Testosterone	5	1.0
19-Nortestosterone	1	<1
17α-Methyltestosterone		0.13
17α-Ethyltestosterone		
17α-Methyl-19-Nortestosterone	0.5	
17α-Ethynyl-19-Nortestosterone	0.03	

TABLE 9. COMPARATIVE ENHANCING RATIOS AS A RESULT OF LOSS OF C-19 (FORMATION OF 19-NOR DERIVATIVE) BY SUBCUTANEOUS INJECTION (SI) AND GAVAGE (G)

Steroid	Enhancing ratio	
	SI	G
Testosterone	0.25	4
17α-Methyltestoterone	1.0	6.5
17α-Ethyltestosterone	62	>100
17α-Ethynyltestosterone	31	40
Androstane-3β,17β-Diol	0.03	0.25
17β-Hydroxyandrostan-3-one	0.2	1.0

TABLE 10. COMPARATIVE ENHANCING RATIOS AS A RESULT OF 17α-METHYL, 17α-ETHYL, AND 17α-ETHYNYL SUBSTITUTION STUDIED BY SUBCUTANEOUS INJECTION (SI) AND GAVAGE (G)

Steroid	17α-methyl		17α-ethyl		17α-ethynyl	
	SI	G	SI	G	SI	G
Testosterone	7.8	16	1	< 1	1	1
19-Nortestosterone	15.6	25	62	25	31	31
19-Nor-17β-Hydroxyandrostan-3-one	7.8	—	2	—	1.0	1.0
17β-Hydroxyandrostan-3-one	0.2	2	—	—	—	—
Δ^5-Androstene-3β,17β-Diol	> 2	< 0.25	—	—	—	—
19-Norandrostane-3β,17β-Diol	> 1	0.1	—	—	> 4	2.0

enhancing ratios were as high as 16 for testosterone in favor of gavage, and the smallest advantage was found for androstane-3β, 17β-diol, which was as high as 5.

Essentially similar enhancing ratios for both the subcutaneous and oral routes were found for all the 17α-alkyl substituted derivatives of 19-nortestosterone and for 17α-methyltestosterone (Table 10). These compounds can also be characterized by the high individual enhancing ratios per se. Contrariwise, no change could be detected for the 17α-ethynyl derivative of 19-nor-hydroxyandrostan-3-one. Values of unity for both the injection and gavage studies were recorded. Striking differences between the oral and injection routes were found in a number of instances, with usually the higher enhancing ratio found for the injection route. Ten-fold differences in favor of the injection route were observed for the 17α-methyl derivatives of the following steroids: 17β-hydroxyandrostan-3-one, Δ^5-androstene-3β,17β-diol, and 19-norandrostane-3β,17β-diol. A similar but less intense effect was recorded for 19-norandrostane-3β,17β-diol and its 17α-ethynyl derivative.

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NON-IODIDE RADIOACTIVITY IN COMMERCIAL SOLUTIONS OF SODIUM RADIO-IODIDE (I^{131})[1]

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ABSTRACT

Some samples of commercially available solutions of sodium radio-iodide (I^{131}) were found to contain radioactive iodine in a form electrophoretically and chromatographically different from iodide. This material (designated "S") was anionic, was not affected by reducing agents in neutral or alkaline solution and its radioactivity could be converted almost completely to iodide by heating or by acidifying the solution. Errors in the estimation of thyroidal uptake of iodide could conceivably occur under certain circumstances if tracer solutions containing appreciable "S" were used. In the more recently available solutions of sodium radio-iodide (I^{131}), some of which contained cysteine hydrochloride, only iodide-I^{131} has been detected.

SEVERAL recent reports (1–3) have directed attention to the occurrence of radioactive components chromatographically distinguishable from iodide in some preparations of sodium iodide^{131}. In using a commercial solution of sodium iodide^{131} as a standard for the identification of labelled iodide in serum subjected to zone electrophoresis in starch blocks it was found that two electrophoretically distinct zones of radioactivity were identifiable, only one of which corresponded to the stable carrier iodide zone. Further study by paper electrophoresis revealed the presence of similar non-iodide radioactive material in several other samples of commercially available radio-iodide solutions. The ease with which the separation of the iodide and the non-iodide radioactive components could be achieved by zone electrophoresis facilitated partial characterization of the foreign material, comparison of the chromatographic and electrophoretic behavior of the anomalous components and the development of simple methods for their transformation into iodide-I^{131}.

METHODS

Electrophoresis on Whatman 3 MM filter paper was done between heavy glass plates by the method of Kunkel (4), barbital buffer, pH 8.6, ionic strength 0.075 being used. Electrophoresis was done for 3 hours at room temperature, at a potential difference of 5 volts per cm. The strips were then dried in air and the distribution of radioactivity de-

Received June 2, 1960.

[1] This investigation was supported by a research grant (A-2585 (C1)) from the Institute of Arthritis and Metabolic Diseases, Public Health Service.

termined by an automatic strip scanning system of conventional type, the proportion of each component being determined by planimetry of the recorded patterns. The cerate-arsenious acid method (5) was used to detect stable iodide and iodate on the paper when these substances had been added to the samples undergoing electrophoresis. Starch block electrophoresis was done on parallel 70 gm. blocks (6); after the blocks had been scanned segments cut from the center of the zones of radioactivity were eluted with a small quantity of water, filtered and the filtrate used for further study. Ascending filter paper chromatography was done using butanol:ethanol:2N ammonia (5:1:2).

Twenty preparations of sodium radio-iodide were examined. These included 10 samples of Iodotope Squibb sodium radio-iodide (I^{131}) oral solution, 8 of Oriodide oral solution sodium radio-iodide (I^{131}) Abbott, 1 sample of Iodotope Squibb for injection and 1 of sodium radio-iodide sterile solution, Abbott. All but one of the solutions studied were obtained between December 2, 1959 and April 27, 1960; the remaining sample was Squibb Iodotope solution examined in January, 1959. Electrophoretic study of the samples was performed 1 day to 3 months after their arrival in the laboratory, but generally this was done within a few days after shipment. Stock solutions were stored at room temperature in their original containers. Most of the data relating to the characterization and isolation of the radioactive substances was obtained using Squibb Iodotope material.

RESULTS

Most of the preparations supplied by both Squibb and Abbott obtained before March, 1960 showed on electrophoresis two major anodally-migrating zones of radioactivity in varying proportions, the material with the greater mobility corresponding in position to iodide. The component migrating more slowly than iodide, designated "S," had a mobility approximately ½ that of iodide. A comparison of the chromatographic and electrophoretic behavior of one preparation is shown in Figure 1; the R_F of the "S" zone in this chromatographic solvent system was slightly greater than that for iodide. Although the electrophoretic mobility of added iodate was only slightly different from that of the "S" material, these substances were widely separated on chromatograms (Fig. 1).

In eight Squibb preparations studied (one sample of January, 1959, and seven in the period December 2, 1959 to March 23, 1960) iodide-I^{131} accounted for 14 to 86% (average 50%) of the total radioactivity, and in six Abbott preparations obtained between December 7, 1959 and February 17, 1960, the iodide-I^{131} radioactivity was 17 to 88% (average 33%) of the total. By contrast in all 3 Squibb solutions obtained in April, 1960, the radioactivity on electrophoresis was present entirely in the iodide zone, and 3 Abbott preparations obtained in March and April, 1960 contained only iodide-I^{131}.

The fraction of the total radioactivity in the "S" zone in those samples which contained it did not seem to be related to the age of the solutions, since some solutions examined within 48 hours of their arrival in the laboratory contained a smaller percentage of the I^{131} as iodide than solutions studied several months after arrival, nor did it seem related to the initial concentration of radioactivity (generally 1 to 5 millicuries/ml.). In some

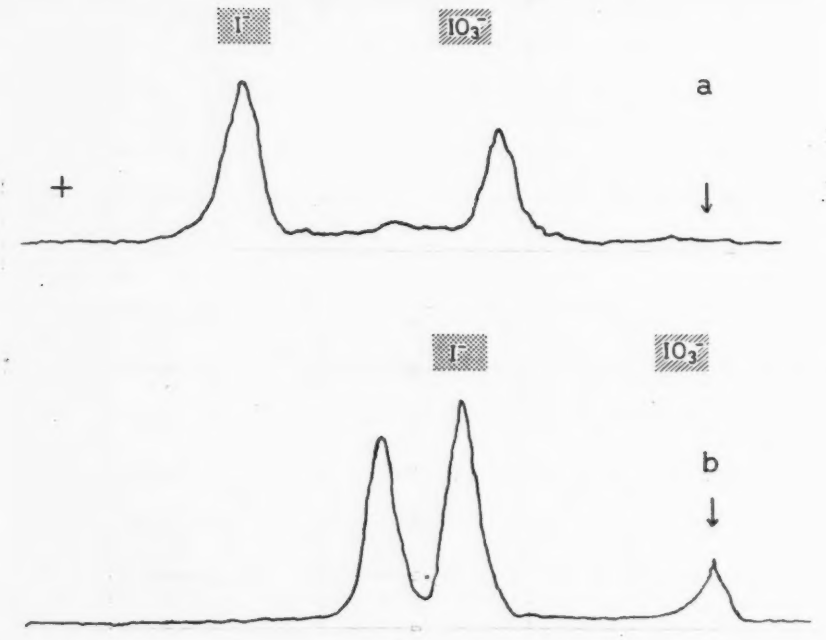

FIG. 1. Scan records of the distribution of radioactive iodine on filter paper strips after electrophoresis (a) and chromatography (b) of a solution of Squibb Iodotope sodium radio-iodide (#75W21). The arrows indicate the origin, and the positions of stable iodide and of iodate are shown. 54% of the total radioactivity was in the iodide position, and most of the remainder was in the zone migrating more slowly than iodide on electrophoresis, and more rapidly on chromatography.

electrophoretic patterns "S" zone radioactivity appeared to consist of several poorly resolved components (e.g. Fig. 2 a, b, c) and this was more apparent on radioautographs of the strips than on the scan records; this is somewhat like the multiple bands other than iodide-I^{131} pictured in the chromatograms of Doctor and Trunnell (1). It is possible that breakdown of "S" material during electrophoresis could account for some of the asymmetry of this zone. A 2-dimensional electrophoretic pattern of a Squibb Iodotope oral preparation is shown in Figure 3; the "S" and iodide bands of radioactivity displayed the same respective mobility in the second direction as in the first, as indicated by the linear arrangement of the spots and the origin. These results suggested that the "S" zone was a component of distinctive mobility and did not arise as a result of iodide adsorption by the paper. "S" material isolated from starch block electrophoresis of several radioiodide preparations and subjected to further electrophoresis on paper maintained its characteristic slower anodal mobility as compared with

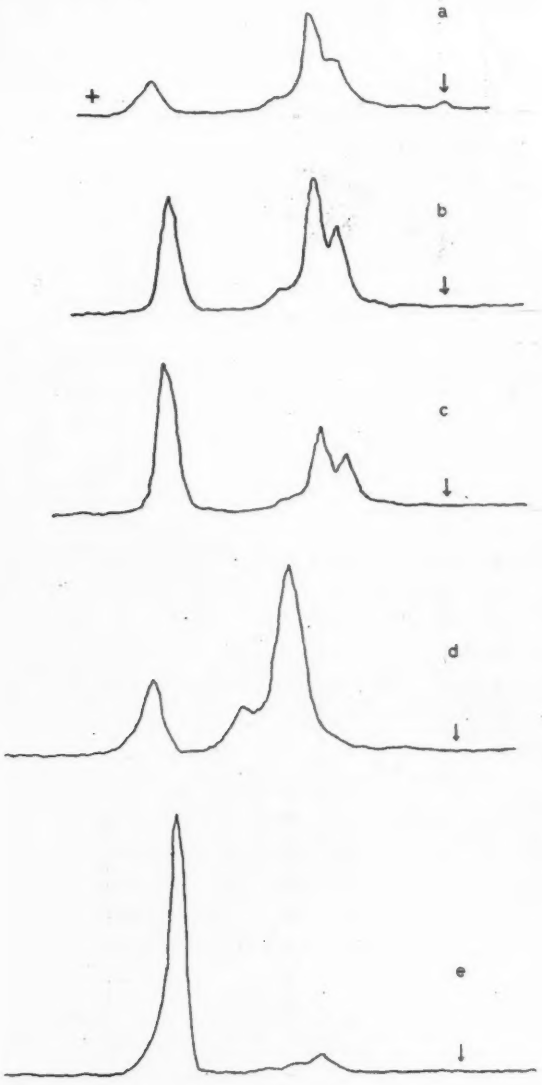

FIG. 2. Electrophoresis of Squibb Iodotope preparations showing distribution of radioactivity. (a) Carrier-free solution; (b) same solution as (a) but heated 10 minutes in boiling H_2O bath before electrophoresis; (c) same solution as (a) but with KI^{127} added to final concentration 63.5 μg. per ml., and then heated 10 minutes in boiling water. (d) Another carrier-free solution, (e) same as (d) but autoclaved before electrophoresis. Iodide-I^{131} accounts for 14% of the total radioactivity in (a), 33% in (b), and 53% in (c); 19% in (d) and 88% in (e).

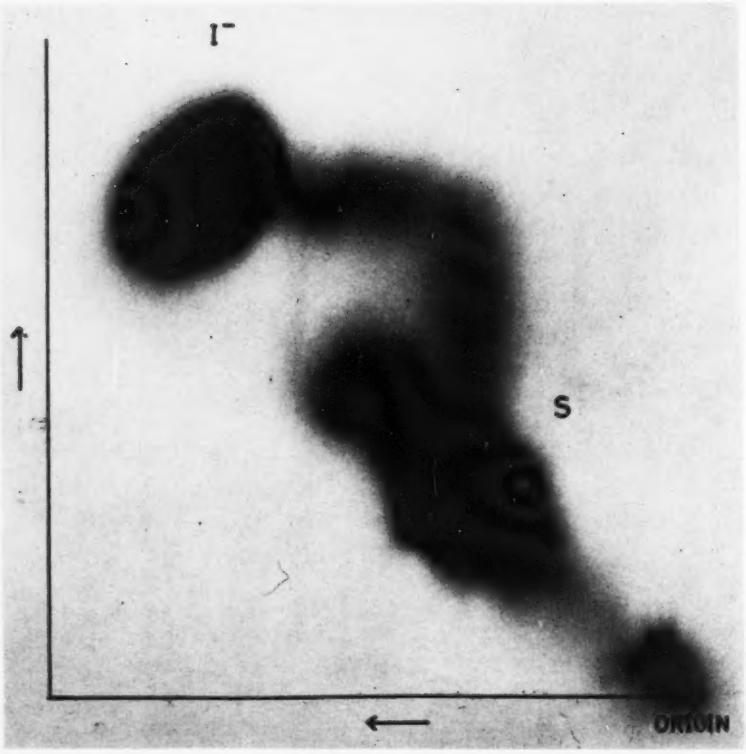

FIG. 3. Radioautograph of 2-dimensional electrophoresis (barbital buffer, pH 8.6 in both directions) of a Squibb Iodotope solution, to which KI carrier had been added. The "S" and iodide zones of radioactivity lie in a line passing through the origin, indicating that the relative mobilities of the major components are the same in both directions. The elongated fainter areas forming a right angle to the right of the iodide spot and above the "S" spot could represent iodide-I^{131} resulting from "S" degradation during the first and second migrations, respectively. The spot just to the upper left of "S" appears to be a distinct component of mobility somewhat greater than "S" (? iodate).

iodide. Under certain conditions, as described below, the instability of "S" we considerable and conversion to iodide was observed.

Thousand-fold aqueous dilution of radioiodide solutions showing both "S" and iodide radioactive zones produced no appreciable change in pattern. Addition of stable iodide to solutions produced relatively small changes in the electrophoretic pattern of radioactivity unless enormous concentrations were achieved. Thus, simultaneous electrophoresis on 3 parallel starch blocks of a Squibb Iodotope solution a) without added carrier, b) containing potassium iodide at a concentration of 12.7 μg./ml. and c) containing potassium iodide at a concentration of 1.27 milligrams

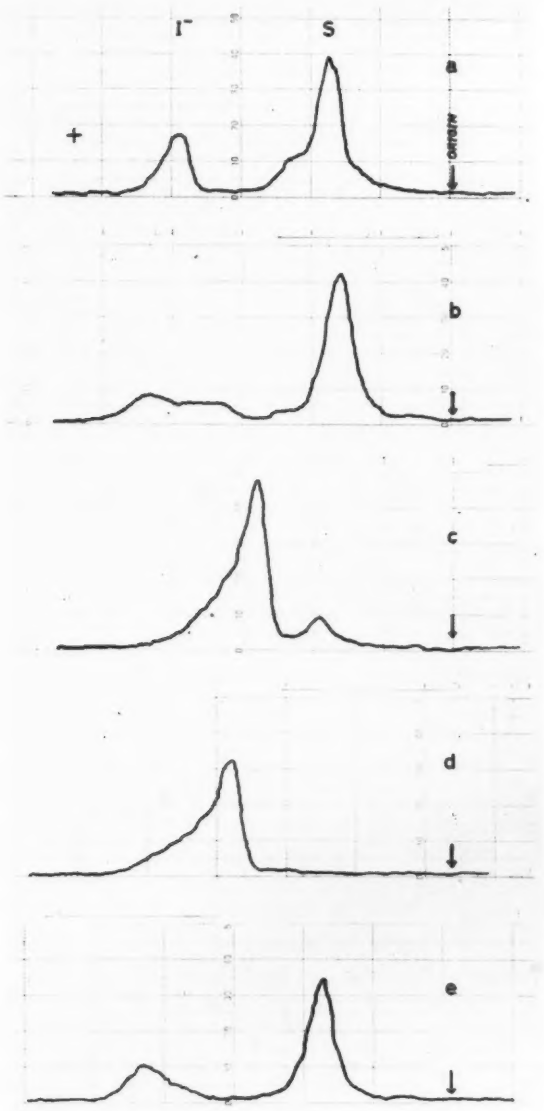

FIG. 4. Effect of pH and reducing agents on iodide-I^{131} content of a Squibb oral Iodotope preparation. Electrophoresis scan patterns are as follows: (a) Carrier-free solution pH 8.6; (b) same as (a) but made 0.8N in NaOH a few minutes before electrophoresis, (c) same as (a) but made 0.7N in HCl for 5 minutes and neutralized to pH 7.5 with NaOH before electrophoresis; (d) same as (c) but an equal volume of 1% Na_2SO_3 was added to the acidified solution prepared as in (c) before neutralization with NaOH; (e) same as (b), but with equal volume of 1% Na_2SO_3 added before NaOH. The percentage of the

per ml., revealed that of the total radioactivity in a, b and c, respectively, 73, 67 and 55% was in the "S" zone. It thus appeared that the occurrence of "S" zone radioactivity could not be dismissed as a tracer artefact, nor could it be explained on the basis of an electro-chemical change in iodide resulting from the electrophoretic procedure.

It was considered extremely unlikely that the non-iodide radioactivity represented either another radioactive isotope of iodine or a different radioactive element, since a) the rate of radioactive decay of the commercial preparations tested conformed to the 8-day half life of I^{131} and b) radioactive "S" component could be converted to material exhibiting the electrophoretic and chromatographic properties of iodide.

Effect of pH. The pH of the original solutions of radioiodide lay between 4 and 8. Electrophoretic patterns in barbital (pH 8.6) and phosphate (pH 7.4 and 6.8) buffers were identical, indicating that the nature of the buffer anion was probably not a factor determining the presence of the 2 radioactive zones. In citrate buffer, pH 3.7, however, the electrophoretic pattern of radio-iodide solutions showing 2 zones at pH 8.6 and of isolated "S" material showed predominantly a single band, corresponding to iodide. Recovery measurements showed that the effect of acid on increasing the proportion of radioactivity as iodide was attributable to a shift of radioactivity from the "S" to the iodide zone, rather than to a loss of radioactivity from the system.

Radio-iodide preparations acidified with mineral acids (HCl or H_2SO_4) to pH 1–3 for a period of 5–20 minutes and then neutralized with NaOH showed a great increase in the proportion of the total radioactivity in the iodide zone of the electrophoretic pattern, 90–100% of the radioactivity behaving as iodide (Fig. 4 c, d, Fig. 5 e), with no loss of radioactivity from the solution. The change induced by lowering the pH occurred rapidly and it also appeared to be irreversible, since the radioactivity in the iodide zone of solutions neutralized after acidification did not seem to decrease even with prolonged standing.

The acid lability of the non-iodide radioactive material in Squibb Iodotope has been commented upon by Taurog (3), and Doctor and Trunnell (1) found less extraneous material when using acid chromatographic solvent systems than with basic solvents.

Effect of temperature. At room temperature carrier-free solutions of radioiodide retained for many days their bizonal electrophoretic character-

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total I^{131} in the iodide zone was: (a) 26%, (b) 27%, (c) 88%, (d) 100%, (e) 25%. The conversion of "S" radioactivity to iodide-I^{131} by acidification (c) and especially by acidification plus a reducing agent (d) is in contrast to the lack of effect of strong alkali (b) and of reducing agents in neutral solution (e). Although the leading zone in (c) and (d) migrated a shorter distance then the iodide zone in other patterns, this zone corresponded in position to added carrier iodide.

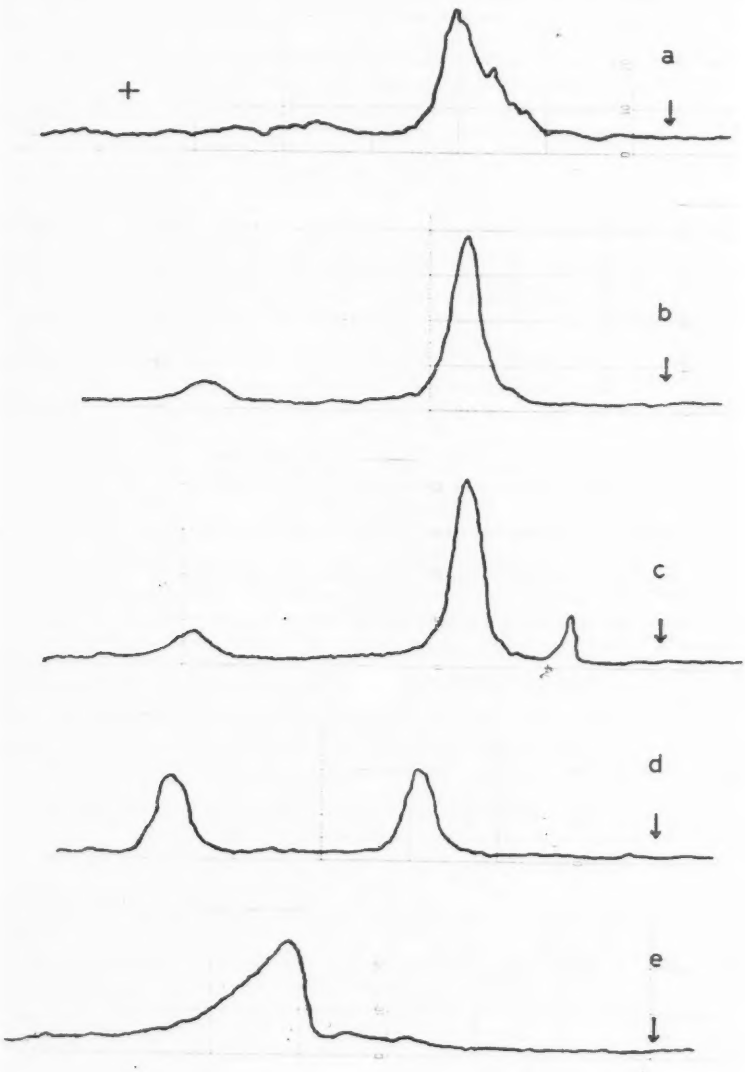

FIG. 5. Effect of pH and heat on radioactive "S" material. "S" material eluted from starch block electrophoresis of a commercial NaI^{131} solution. Electrophoresis scan patterns; 2 experiments, (a) and (e) were done simultaneously, and (b), (c) and (d) were done together but at another time. (a) "S" material, with added KI to a concentration of 100 μg. per ml. (b) "S" carrier-free, heated 5 minutes in boiling water; (c) same as (b) but with KIO_3 added before heating, (d) same as (b) but with KI added (final concentration 100 μg./ml.) before heating, (e) same as (a) but allowed to stand at room temperature with equal volume 1N HCl before neutralization and electrophoresis. The proportion of the total I^{131} as iodide was: (a) none, (b) 11%, (c) 12%, (d) 42%, (e) >95%.

istic with relatively minor quantitative changes. However, heating the radioiodide preparations or isolated "S" material had appreciable effects on the patterns, tending to decrease the quantity of radioactivity in the "S" zone and commensurately to increase the iodide zone activity. Heating neutral aqueous solutions in the boiling water bath for 5 to 10 minutes produced a perceptible change in composition, while autoclaving (115 lb./sq. in. for 30 minutes) almost completely converted "S" radioactivity to iodide (Fig. 2).

Although the presence of stable iodide had little effect on patterns at room temperature, it greatly accelerated the conversion of "S" to iodide produced by heating (Fig. 5 b, c). Commercial oral solutions that were autoclaved and then contained predominantly iodide-I^{131} seemed fairly stable, and even after some days at room temperature contained approximately 90% of the total radioactivity as iodide.

The effect of heat and lowering pH were to some degree additive; heating a solution at pH 4.6 for 10 minutes in the boiling water bath resulted in considerably more conversion of "S" radioactivity to iodide than when the pH was 8.6.

Effect of reducing agents. The addition of sodium sulfite or bisulfite to a final concentration of 1% did not modify the electrophoretic pattern of iodide-I^{131} solutions rich in "S" zone radioactivity, while sodium hydrosulfite was effective in converting most of the radioactivity to iodide. Since the pH of the sodium hydrosulfite solution was approximately 3, the conversion of "S" to iodide-I^{131} might have been due to the acid-instability of "S." There was some suggestion in several experiments that at pH approximately 3 the addition of sulfite produced a more complete conversion of "S" zone radioactivity to iodide-I^{131} than occurred with acidification alone (Fig. 4 c, d), but at pH below 1 conversion by acid alone seemed complete. The effect of cysteine was studied, since Doctor and Trunnell (1) had found that a high concentration of cysteine eliminated extraneous I^{131} material from commercial solutions of radioiodide. Their observation was confirmed, the addition of cysteine hydrochloride (final concentration 0.2–0.5%) to preparations containing "S" radioactivity resulting in material showing, on electrophoresis, a radioactive band only in the iodide position. It should be pointed out, however, that the pH of a 0.2% solution of cysteine hydrochloride is 2.2, and if the cysteine hydrochloride solution was neutralized (NaOH) to pH 6 a few minutes before adding it to the I^{131} preparation the resulting mixture was not effective in the conversion of "S" to iodide, and therefore, it is possible that the effectiveness of cysteine in achieving electrophoretic and chromatographic purity in iodide-I^{131} solutions may be a consequence not only of its reducing action but also its acidity.[2]

[2] Although cysteine in neutral solution is readily oxidized in air it seems unlikely that oxidation was complete before the freshly neutralized cysteine solution was added to the I^{131} preparation.

Other properties of "S." The anionic character of the "S" component suggested by electrophoretic anodal movement was confirmed by its complete removal from aqueous solution by anion-exchange resin (Amberlite IRA-400). The radioactivity was soluble in trichloroacetic acid, was not extractable from aqueous solution by chloroform, nor did the addition of stable iodide and acidification result in chloroform solubility (indicating that the "S" material was not predominantly iodate). Radioactivity of solutions containing both "S" and iodide-I^{131} was rendered chloroform soluble on acidification and addition of iodate, to the same extent as electrophoretically pure iodide-I^{131} treated in the same way; it is likely, however, that the low pH required for the formation of iodine from iodide and iodate may have led to the conversion of all the "S"-I^{131} to iodide.

The Abbott and Squibb radioactive iodide preparations contained benzyl alcohol (0.9%) and a reducing agent. To explore the possibility that "S" material might be the result of some reaction between iodide and the alcohol or sulfite, to solutions of electrophoretically pure iodide-I^{131} eluted from a starch block were added benzyl alcohol (final concentration 3%), sodium sulfite (1%) or both, and the solutions were kept at room temperature for several days; in no case did "S" zone radioactivity appear.

DISCUSSION

The presence of a substantial fraction of the total radioactive iodine in some commercial solutions of sodium radio-iodide (I^{131}) in a form other than inorganic iodide is in agreement with the reports of radioactive impurities detected by chromatographic methods (1–3). The nature of the foreign material is unknown, but its striking characteristics are: 1) anionic character, 2) convertibility of its labelled iodine to iodide-I^{131} by heat or acidification, and 3) non-reducibility in neutral or alkaline solutions. The effect of increased concentration of stable iodide on facilitating the "S" to iodide-I^{131} conversion induced by heat or acidification suggested the possibility of an exchange of stable iodide with "S" I^{131} that occurred very slowly at room temperature or in neutral or alkaline solution. Application of the cerate-arsenious acid reaction to paper electrophoretograms of solutions which had been heated with stable iodide so that all the radioactivity was in the iodide band failed to reveal the presence of iodine in areas other than the iodide zone, and in particular not in the "S" zone position; this suggested that if an exchange reaction of inorganic I^{127} with S-I^{131} did occur, the "S" component must have been present in very low concentration.

These properties provide possible explanations from some of the observations that have been made with respect to these solutions of radio-iodide The effect of cysteine hydrochloride in eliminating radioactive bands other than iodide may be at least in part related to the acidity of such solutions, since a similar result could be attained by acidifying the I^{131} solution with mineral acids particularly if an inorganic reducing agent was present.

Doctor's (2) observation that the administration of stable iodide increased the thyroidal uptake of radioactive iodine in chicks injected with I^{131} obtained from the non-iodide portion of filter paper chromatograms of aged commercial solutions of sodium radioiodide might be explained on the basis of an exchange between stable iodide and the radioactive iodine.

The smaller thyroidal uptake of radioactive iodine in rats given Squibb oral solution of sodium radio-iodide (I^{131}) (3) and in chicks given extraneous I^{131} material separated from commercial NaI^{131} preparations (2) as compared with animals given pure NaI^{131}, suggests the possibility of error in using the commercial impure iodide solutions as a tracer for iodide in biological and chemical work. One might expect that in man the low pH of the gastric contents might result in some conversion of "S" radioactivity to iodide-I^{131} if "S" containing preparation of I^{131} were taken orally, while in human studies in which I^{131} was given parenterally solutions sterilized by autoclaving (and therefore containing little "S" material) would almost certainly be used; for these reasons one might expect that glandular uptake of orally or parenterally administered tracer I^{131} in man might be little influenced by the presence of impurities of the "S" type in some commercial I^{131} preparations. However, gastric achlorhydria or neutralization of gastric acidity by food and other agents could conceivably make the conversion of "S" to iodide incomplete. We have recently observed 3 cases of hyperthyroidism in all of which the values of 24-hour glandular uptake of orally administered radioiodine, using a commercial preparation containing appreciable "S" material were normal or borderline normal (31, 45 and 57%). When the uptake studies in these cases were repeated a few days later using autoclaved samples of the tracer solutions, the uptakes were 65, 71 and 83% of the administered dose respectively. These results suggest the possibility that the oral use of I^{131} preparations containing non-iodide impurities may result in lower glandular uptakes of radioactivity. One might anticipate that such erroneously low values for glandular uptake would be encountered more frequently in cases of hyperthyroidism since gastric hypoacidity or anacidity has been observed in approximately one-third of such cases (7).

Both Abbott and Squibb oral sodium radioiodide solutions obtained in April and May, 1960 contained, according to the label, 0.2% cysteine hydrochloride. Electrophoresis and chromatography of these preparations has revealed only a single zone of radioactivity, corresponding to iodide. The chromatographic and electrophoretic "purity" thus achieved may also be attained by adding acid, a small quantity of sulfite and then neutralizing, or by autoclaving the solutions. The sulfite or bisulfite addition would seem warranted in addition to acidification since more complete conversion of non-iodide radioactivity to iodide was observed under these circumstances and also because it would hinder the oxidative changes iodide is known to undergo in neutral or alkaline solution (8).

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PRODUCTION OF ANOVULATORY, STERILE RATS BY SINGLE INJECTIONS OF TESTOSTERONE PROPIONATE[1]

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ABSTRACT

The current investigation studies the effect of a single injection of androgen in the prepubertal female rat on subsequent development of the endocrine system. A single subcutaneous injection of 1.25 mg. of testosterone propionate was administered to 2, 5, 10 or 20 day old female rats. The animals were allowed to mature to 100 days of age and then were either autopsied or tested for fertility. At autopsy, adrenal and pituitary weights were significantly larger than littermate controls in rats treated at 2 or 5 days of age. No difference in adrenal, pituitary or uterine weight was observed in rats treated at 10 or 20 days of age. Ovarian and uterine weights were markedly smaller in animals injected at 2 or 5 days of age, but not in animals treated at 20 days of age. The 10 day injected group assumed an intermediate position. Corpora lutea were absent from the ovaries of the 2 and 5 day injected groups and from 4 of 10 rats treated at 10 days of age when autopsied at 100 days of age. Breeding studies revealed that administration of androgen at 2 or 5 days of age induced permanent sterility in all animals and in 4 of 10 rats treated at 10 days of age. All animals injected at 20 days of age bore normal litters. It is suggested from these experiments that an androgen-sensitive period exists between birth and the tenth day of age.

IT IS well recognized that prepubertal treatment of female rats with androgen results in subsequent infertility (1, 2, 3, 4). However, such investigations invariably have employed either testicular implants or multiple injections of androgen given over prolonged periods of time. This type of experimentation makes it difficult to ascertain if an age of androgen sensitivity exists during the early postnatal developmental period comparable to that observed in the female mouse, in which single injections of testosterone propionate to 5 day old animals resulted in permanent sterility. In contrast, fertility was not influenced in mice receiving androgen at 20 days of age, whereas mice treated at 10 days of age assumed an intermediate position (5). The current study was designed to evaluate the effect of a single injection of androgen at a definite time in development, and the effect and duration of effect on the developing gonad of the rat. This investi-

Received June 10, 1960.

[1] Supported by grants NSF-G2226 from the National Science Foundation and RG-5496 from the United States Public Health Service.

gation also permits a comparison of the ages of androgen sensitivity in the rat and mouse, 2 species which ovulate spontaneously. Portions of this study have been reported previously in abstract form (6).

MATERIALS AND METHODS

The Sprague-Dawley rats used in these experiments were raised in our laboratory in a temperature-controlled, artificially illuminated room. A diet of Purina rat pellets supplemented by a mixture of cod liver oil and Mazola oil on bread once a week was fed the animals through the period of experimentation.

Intact female rats of 2, 5, 10 or 20 days of age were given a single subcutaneous injection of 1.25 mg. of testosterone propionate in oil. The single injection permitted a minimum of disturbance to the nursing animals which were kept with their mothers until weaning. The rats selected were of such a number as to provide an approximate equal distribution of littermates between control and injected groups. All rats were either autopsied or tested for fertility at 100 days of age.

Body weights were recorded only at the time of autopsy. The rats were sacrificed with ether and the organs were dissected under a binocular microscope. Fresh weights of ovary, uterus, adrenal and pituitary were obtained on a chainomatic balance immediately following dissection and a comparison of such weights was made between the control and injected littermates. Both ovaries and a segment of uterus of each animal were placed in Bouin's fixative for 24 hours. Individual tissues for each animal in the control and experimental groups were sectioned in paraffin and stained with hematoxylin and eosin.

Fertility studies were begun at 100 days of age by placing littermate control and treated rats from one litter with a male. The time between admitting the male and littering was recorded as well as the litter size, sex distribution, and number and weight of the young carried to weaning at 22 days of age.

Litters were reduced to 8 animals at 3 days of age to permit more uniform growth.

RESULTS

The effects of administration of 1.25 mg. of testosterone propionate at 2, 5, 10 and 20 days of age on body and organ weights are shown in Table 1. Autopsy at 100 days of age revealed an increased body weight in animals injected at 2 or 5 days of age as compared with body weights of the littermate controls. In contrast, no change in body weight was observed at autopsy between rats treated at 10 and 20 days of age and their littermate controls. Adrenal and pituitary weights were significantly larger ($P > .05$), even when expressed as mg./100 gm. body weight, in rats treated at 2 and 5 days of age, but if treatment was delayed until 10 or 20 days of age such alterations in organ weight were not observed. Ovarian weights in rats injected with androgen at 2, 5, or 10 days of age were found to be approximately one-half that of the littermate controls, whereas ovarian weights of animals treated at 20 days of age did not differ from the control weights. Similarly, uterine weights were significantly smaller than normal in rats treated at 2 or 5 days and autopsied at 100 days of age. The uteri of animals treated at 10 or 20 days of age were comparable to their littermate controls at autopsy.

TABLE 1. AVERAGE ACTUAL AND RELATIVE ORGAN WEIGHTS OF 100-DAY-OLD RATS FOLLOWING A SINGLE INJECTION OF TESTOSTERONE PROPIONATE AT 2, 5, 10 AND 20 DAYS OF AGE

Treatment	No. of rats	Age injected (days)	Body wt. (gm.)	Adrenal wt.	Pituitary wt.	Ovary wt.	Uterus wt.
				mg./100 gram body weight			
Control	6	—	171.8	25.1 (43.1±2.8)†	4.83 (8.29±0.6)	30.8 (52.7±1.7)	238.5 (409.8±49.5)
Androgen	6	2	189.5	28.9 (54.9±2.3)	6.42 (12.2 ±0.3)	14.2 (26.8±1.0)	139.5 (264.0±11.6)
Control	8	—	187.8	28.4 (53.4±3.2)	5.75 (11.2 ±0.2)	30.2 (56.7±2.7)	165.4 (310.7±32.4)
Androgen	7	5	232.1	31.1 (72.2±4.2)	6.75 (12.7 ±0.4)	12.3 (28.6±1.3)	101.2 (235.6±9.4)
Control	10	—	202.4	34.7 (70.2±2.5)	5.78 (11.7 ±0.2)	33.6 (68.0±2.4)	168.4 (340.4±27.7)
Androgen	8	10	207.6	34.2 (70.9±5.3)	5.12 (10.6 ±0.7)	24.5 (50.9±7.2)	170.4 (352.9±22.4)
Control	6	—	234.8	31.9 (74.6±3.2)	6.26 (14.7 ±0.3)	29.5 (69.3±2.5)	178.4 (419.1±30.1)
Androgen	6	20	232.5	31.0 (72.1±3.8)	6.06 (14.1 ±0.2)	29.1 (67.6±1.8)	185.9 (432.3±27.2)

† Numbers in parenthesis represent average actual organ weights ± standard error.

Ovarian and Uterine Histology: The normal ovary at 100 days of age contained several generations of corpora lutea as well as follicles in various stages of development (Fig. 1). In contrast, corpora lutea were absent from the ovaries of rats treated with androgen at 2 or 5 days of age, although numerous large vesicular follicles were present and interstitial tissue hyper-

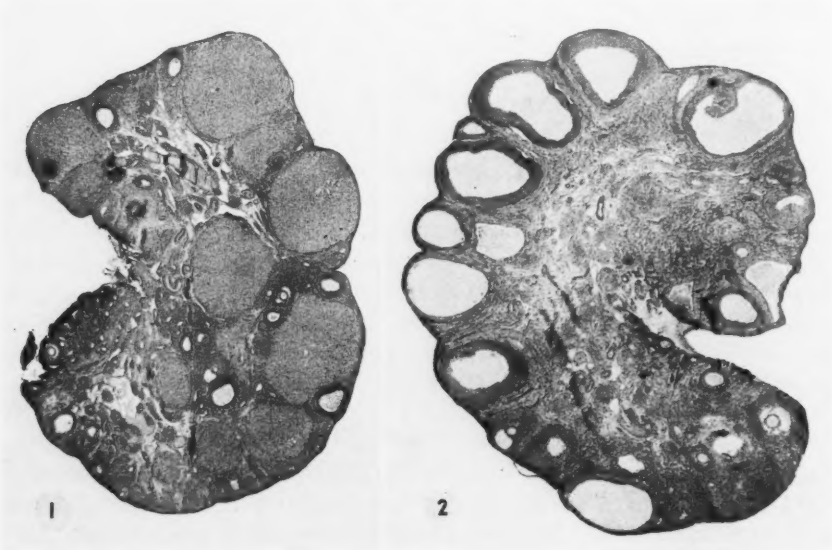

FIG. 1. Normal ovary ×17.

FIG. 2. Ovary of rat treated with 1.25 mg. of testosterone propionate at 5 days of age and autopsied at 100 days of age; ×20.

TABLE 2. EFFECT OF AGE AND TESTOSTERONE PROPIONATE (1.25 MG.) ON REPRODUCTIVE PERFORMANCE OF PREPUBERTAL RATS

Treatment	No. of rats	No. of pregnancies	Days in breeding to first litter, avg. (range)	Avg. No./ litter	Avg. weaning wt. (gm.)
Control	10	10	28 (22–35)	8.8	47.9
T. P. at 2 days	10	0	Infertile	—	—
Control	10	10	31.6 (22–40)	8.6	42.7
T. P. at 5 days	10	0	Infertile	—	—
Control	12	12	28.7 (22–38)	9.1	45.7
T. P. at 10 days	10	6	29.3 (22–41)	8.9	48.2
Control	14	14	33.3 (22–46)	9.3	46.3
T. P. at 20 days	16	16	34.7 (22–48)	9.1	47.5

trophy was evident (Fig. 2). Androgen administration at 10 days of age had a less drastic effect on ovarian structure, with only 4 of 10 animals failing to show corpora lutea at 100 days of age. All ovaries of rats treated at 20 days of age were normal at autopsy. The uteri of rats treated with androgen at 2 or 5 days of age failed to exhibit the changes in endometrial histology normally associated with the cyclic fluctuations in ovarian estrogen secretion. The endometrial stroma was compact and nonedematous and it contained few glands, whereas the epithelium was hypertrophied and composed of tall columnar cells. The myometrium was comparable in appearance to that of the normal uterus. A similar uterine structure was observed in the 4 animals which failed to form corpora lutea following injection at 10 days of age. However, no difference in uterine structure was discernible in the remainder of this latter group (10 day injected animals) nor in any of the rats treated at 20 days of age.

Breeding Experiments: Fertility was tested in both littermate control and androgen-treated rats at 100 days of age. All untreated control animals delivered litters within 22–48 days after being placed with the male. In contrast, single injections of testosterone propionate to rats at 2 or 5 days of age resulted in subsequent infertility. Although these animals were kept with proven males for 100 days, no pregnancies were observed. A less damaging effect on fertility occurred if androgen-treatment was delayed until 10 days of age, as only 4 of 10 rats failed to produce litters.

Those rats which were fertile delivered their litters at approximately the same time as their littermate controls, had approximately the same number of pups per litter, and exhibited good lactation as evidenced by litter weight at weaning. Testosterone propionate injections at 20 days of age failed to diminish fertility (Table 2).

DISCUSSION

It is apparent from these studies that a period of steroid sensitivity exists in the female rat between birth and the tenth day of age, during

which administration of androgen will result in subsequent infertility. These results demonstrate that the androgen-sensitive age period in the rat is similar to that shown previously for the mouse. It was originally proposed by Pfeiffer (1) that the mechanism by which androgen induced sterility, when administered to the prepubertal rat, was by "masculinization" of the pituitary so that a permanent imbalance in gonadotropin secretion resulted. Pfeiffer suggested from his experiments that the pituitary of the newborn rat was undifferentiated. If it differentiated in the presence of androgen only follicle stimulating hormone was elaborated, whereas differentiation in the presence of estrogen resulted in the subsequent elaboration of both follicle stimulating (FSH) and luteinizing hormone (LH). Since the time of these early experiments it has become apparent that the pituitary of the androgen sterilized rat elaborates both gonadotropins. Not only are vesicular follicles present in the sterile rat ovary, but estrogen is secreted as evidenced by the persistence of a cornified vaginal mucosa. Furthermore, it is the constant estrogen secretion which not only produces the persistent vaginal cornification in rats of the current study, but also could explain the resultant hypertrophy of the adrenal and pituitary glands. Thus the particular malfunction in adenohypophyseal gonadotropin secretion is not the inability of this gland to secrete luteinizing hormone but rather the absence of the ovulatory discharge of luteinizing hormone. This suggests another possible explanation for the mechanism by which androgen acts to induce sterility. The hypothalamus has been shown to exert a controlling influence over the ovulatory discharge of luteinizing hormone. It may be that androgen administration during infancy alters hypothalamic-hypophyseal interrelationships by rendering the hypothalamic areas responsible for the ovulatory discharge of gonadotropin refractory to intrinsic activation. As such, the proper impetus for the ovulatory release of gonadotropin is not supplied to the adenohypophysis and sterility ensues. A further connotation of this hypothesis would be that it is not the pituitary which is undifferentiated at birth, but rather the regions of the hypothalamus controlling gonadotropin secretion. In a subsequent series of experiments we have shown that the adenohypophysis of the sterile rat will respond to extrinsic hypothalamic activation by the discharge of gonadotropin to induce ovulation (7).

The difference in body weights at autopsy between the littermate controls and the experimental animals of the 2 and 5 day injected groups might be attributed to a difference in metabolism between the sterile, persistent estrous rat, and the normal rat. Furthermore, although the protein anabolic action of androgens is well recognized, this in itself would not seem to account for the body weight differences, since animals injected at 10 or 20 days of age were within the weight range of their littermate controls at autopsy. Ovarian weight differences may be explained by the

absence of luteal tissue in the ovaries of animals treated at 2 or 5 days of age, and 4 of 10 animals injected at 10 days of age.

Acknowledgments

The author wishes to express his appreciation for the technical assistance rendered by Miss Theresa Mangold and Mr. Leroy Brown during this study.

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EVIDENCE THAT THE HYPOTHALAMUS IS RESPONSIBLE FOR ANDROGEN-INDUCED STERILITY IN THE FEMALE RAT[1]

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ABSTRACT

The data presented in the current study suggest that the sterility which ensues from androgen treatment of prepubertal female rats is not the result of malfunction of the adenohypophysis. Rather, the pituitaries of such animals will respond to electrical stimulation of the hypothalamus by discharging sufficient gonadotropin to cause ovulation. It is further suggested from these experiments that there are two regions within the hypothalamus which control adenohypophyseal gonadotropin secretion: (a) a region responsible for activating the cyclic discharge of ovulating hormone: the anterior preoptic (suprachiasmatic) area of the hypothalamus, and (b) a region independently responsible for the tonic discharge of luteinizing hormone in a sufficient quantity to cause estrogen secretion but not ovulation: the arcuate-ventromedial nuclei complex of the median eminence. Evidence is presented which suggests that prepubertal androgen treatment deleteriously alters the function of the "ovulation controlling" preoptic region of the hypothalamus.

INTRODUCTION

PREVIOUS studies have demonstrated a period of steroid sensitivity in the female rat between birth and the tenth day of age during which a single injection of androgen will result in permanent infertility (1). It was originally proposed by Pfeiffer that such sterility was the consequence of a deleterious effect of androgen on the adenohypophysis, resulting in a gland that secreted only follicle stimulating hormone (2). Since the time of these early observations it has become apparent that the pituitary of the androgen-sterilized rat elaborates both follicle stimulating and luteinizing hormone. Not only are vesicular follicles present in the sterile rat ovary but estrogen is secreted as evidenced by the persistence of a cornified vaginal mucosa, cystic enlargement of the uterine endometrial glands and hypertrophy of the ovarian interstitial tissue (1, 3). Seemingly, the particular adenohypophyseal malfunction is a failure to release sufficient gonadotropin to cause ovulation, a phenomenon generally held to be regulated by the hypothalamus. This suggests either that the pituitary of the androgen-sterilized rat is refractory to hypothalamic activation or, more likely, that the malfunction in the ovulatory mechanism is inherent

Received June 10, 1960.

[1] Supported by grant RG-5496 from the United States Public Health Service.

within the hypothalamus itself. Some support for the latter hypothesis is offered by the observations of Harris and Jacobsohn (4) and Martinez and Bittner (5) that male hypophyses transplanted beneath the median eminence of hypophysectomized female rats would restore normal estrous cycles. Apparently, the sex difference in gonadotropin secretion is not resident within the adenohypophysis as such, but a higher neural level.

The current studies were designed to answer two questions: (a) will the pituitary of the anovulatory female rat respond to stimulation of the hypothalamus by the release of sufficient gonadotropin to cause ovulation and (b) if so, can a specific region of the hypothalamus be implicated as the site of the deleterious androgen action? The results of these experiments have been presented previously in abstract form (6).

MATERIALS AND METHODS

Five-day-old Sprague-Dawley female rats were given a single subcutaneous injection of 1.25 mg. of testosterone propionate (in oil) and then permitted to mature to a weight of 230–250 gms. (4–5 months of age). This experimental regime was chosen since it had been shown previously to induce permanent sterility in the female rat (1).

Following treatment, the rats were maintained in an artificially illuminated, temperature controlled room, on a diet of Purina rat pellets supplemented by a mixture of cod-liver oil and Mazola oil on bread once a week. During the three weeks prior to experimentation, daily vaginal smears were taken to establish the persistence of a cornified vaginal mucosa. This phenomenon can be correlated with the presence of large ovarian vesicular follicles and the absence of corpora lutea and may thus be used as a criterion of sterility; however, laparotomies were performed periodically in randomly selected rats exhibiting persistent vaginal cornification to confirm the absence of corpora lutea.

At the time of experimentation, all rats were given an intraperitoneal injection of 25 mg./kg. of Nembutal. This dosage produced sufficient sedation to permit orientation of the animal in a stereotaxic apparatus, but did not inhibit ovulation in the normal cyclic rat, and it eliminated the problem of hypothalamic refractoriness due to anesthesia. Following procainization of all areas to be subjected to surgery, and orientation of the animal in the stereotaxic apparatus, bipolar concentric electrodes were placed, bilaterally, in various regions of the hypothalamus. The stimulation parameters chosen for this study were 2–3 volts (80–100 microamperes) square wave pulses delivered by a Grass stimulator at a frequency of 100/sec. with a duration of 0.5 msec. for 15 sec. on/off periods over a total of 15 minutes. Such parameters have been used previously by Critchlow (7) and Everett (8) to induce ovulation in the Nembutal-blocked rat. All voltage and current values used for stimulation were monitored by an oscilloscope previously calibrated for such determinations.

Twenty-four hours after stimulation, the rats were sacrificed with ether and the fallopian tubes were examined for the presence of ova and the ovaries for corpora lutea. To confirm the autopsy results, both ovaries of each animal were placed in Bouin's fixative, paraffin-embedded, serially sectioned and stained with hematoxylin and eosin. The presence of newly formed corpora lutea, correlated with the previously identified tubal ova, served as the criterion of an ovulatory discharge of gonadotropin in response to hypothalamic stimulation.

Similarly all brains were perfused and fixed with 10% formalin, frozen-sectioned and stained with thionine for verification of electrode placements. The additional techniques used in this study will be described with the results of these experiments.

RESULTS

To establish that the adenohypophysis of the sterile rat can respond to hypothalamic stimulation, areas were selected for study which had previously induced ovulation when stimulated, namely the arcuate and ventral medial nuclei (7). However, as is shown in Table 1, electrical stimulation of these median eminence structures failed to cause ovulation in any of 8 sterile rats (Fig. 1). Although these results would superficially tend to support the hypothesis that the pituitary of the androgen-sterilized rat

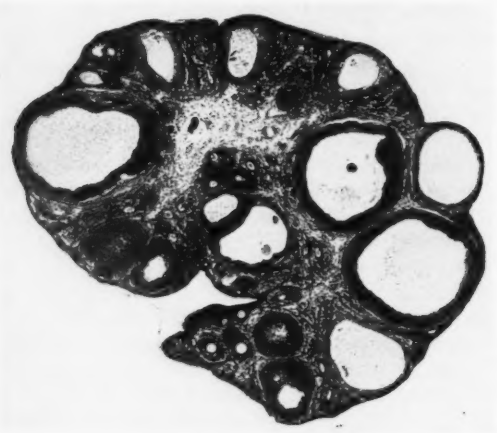

FIG. 1. Ovary of an anovulatory-persistent-estrous rat following electrical stimulation of the median eminence. ×25.

is refractory to hypothalamic activation, a second alternative also deserved consideration. The constant levels of estrogen secreted by the sterile rat ovary might so affect adenohypophyseal physiology as to prevent normal storage of gonadotropin. Thus even though the adenohypophysis was activated by hypothalamic stimulation, gonadotropin was not present in sufficient concentration to cause ovulation. Evidence that the sterile rat pituitary does respond to hypothalamic stimulation by discharging luteinizing hormone is afforded by the increase in uterine weight which occurs 24 hours after such stimulation. Although this is only indirect evidence for the release of LH, the second hypothesis warranted further investigation. Thus a second group of 6 anovulatory rats was given single injections of progesterone in a dosage calculated to interrupt the persistent vaginal cornification (2 mg. s.c. in oil), and presumably the secretion of LH. Such treatment generally induced 2–3 days of diestrus followed by a single day of proestrus. When no further treatment was given, all animals resumed the previous persistent estrous condition. If, however, the ventromedial-arcuate complex was stimulated electrically on the day of vaginal proestrus

TABLE 1

Treatment	No. animals	Body wt. (gms.)	Relative organ wts. mg./100 gm. B. wt.			
			Pituitary wt.	Adrenal wt.	Ovarian wt.	Uterine wt.
Normal Control	10	202.4	5.78	34.7	33.58	168.4
Sterile Control	7	225.2	5.53	35.0	11.75	107.2
Sterile plus median eminence stimulation	8	239.2	5.30	32.4	12.09	113.04
Sterile plus 2 mg. progesterone	8	241.5	5.44	29.35	12.50	107.94
Sterile plus 2 mg. progesterone-hypothalamic stimulation. *No Ovulation*	34	246.5	5.36	30.0	11.20	114.30
Sterile plus 2 mg. progesterone-hypothalamic stimulation. *Ovulation*	11	265.2	5.29	31.7	20.00	135.70

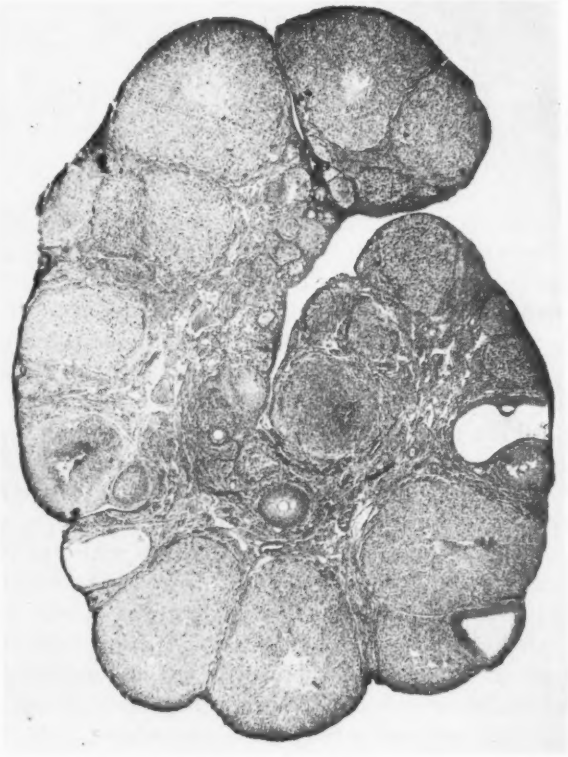

FIG. 2. Ovary of an anovulatory-persistent-estrous rat primed with 2 mg. of progesterone in which electrical stimulation of the median eminence induced ovulation. Note the completeness of luteinization. ×25.

FIG. 3. Ovary of an anovulatory-persistent-estrous rat primed with 2 mg. of progesterone but in which hypothalamic stimulation was not performed. ×25.

all animals ovulated within 24 hours (Table 1, Fig. 2). Progesterone alone failed to cause either luteinization or ovulation of any of the numerous ovarian vesicular follicles (Table 1, Fig. 3).

Once assured that ovulation could be induced in the progesterone-primed sterile rat, a more critical study was undertaken to determine the specific hypothalamic regions in which stimulation would induce ovulation. As is shown in Figure 5, electrical stimulation of the medial or lateral preoptic areas, dorsal to the optic chiasm or in the region of the median forebrain bundle, failed to induce ovulation in 17 of 18 rats. In the one positive response, an incomplete or partial ovulation occurred. Only 2 tubal ova could be identified at autopsy and they were correlated with the presence of 2 newly formed copora lutea. Other ovarian follicles exhibited partial luteinization but contained trapped ova (Fig. 4). In contrast, stimulation of the medial hypothalamic regions immediately rostral or caudal to the arcuate and ventral medial nuclei induced ovulation in 10 of 13 rats (Figs. 5, 6). Stimulation of regions lateral to the ventral medial nuclei or of the medial mamillary nuclei did not induce ovulation in any of the fifteen cases studied (Figs. 5, 6). Thus, in progesterone-primed, anovulatory rats, the hypothalamic areas which would promote an ovulatory discharge of gonadotropin when stimulated could be delineated as extending just rostral and caudal,

FIG. 4. Ovary of a progesterone-primed, anovulatory-persistent-estrous rat which exhibited only partial ovulation following electrical stimulation of the medial preoptic area. Note the presence of two newly formed corpora lutea and the partial luteinization of the large vesicular follicles. These latter follicles contained trapped ova. ×25.

but not lateral, to the ventral medial nucleus. The antero-posterior extent of this positive region is represented in sagittal section in Figure 7.

DISCUSSION

These results suggest that the adenohypophysis of the androgen-sterilized rat can function normally, provided: (a) proper gonadotropin storage is permitted and (b) an impetus for its release is supplied by the hypothalamus. We have interpreted the failure of the persistent-estrous rat to ovulate on artificial hypothalamic stimulation as due to insufficient pituitary gonadotropin stores. When such animals are primed with progesterone they readily ovulate in response to such hypothalamic stimulation. Presumably progesterone permits sufficient gonadotropin to be accumulated in the adenohypophysis to cause ovulation when released. Segal has reported that the pituitary gonadotropin content of the androgen-sterilized rat is comparable to that of the normal male rat (9). However, his assays are based on glands of 60-day-old rats which have exhibited only

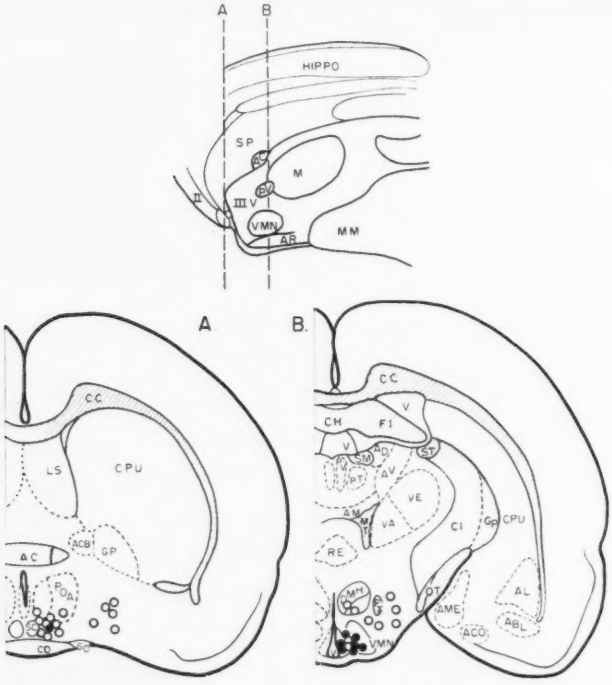

FIG. 5.

2–3 weeks of persistent vaginal cornification, whereas the animals employed in these studies have undergone an additional 60–120 days of persistent estrus (120–180 days of age). It is probable that the pituitary, when subjected to prolonged estrogen stimulation would be depleted of its stored gonadotropin. Preliminary assay data of the pituitary LH content of progesterone-primed persistent estrous rats suggest a twofold increase in content (unpublished observations).

A second mode of progesterone action also deserves consideration. Everett (10) has shown that progesterone will advance ovulation 24 hours in the normal cyclic rat provided this steroid is administered on the last day of diestrus. Furthermore, Kawakami and Sawyer (11) have observed that progesterone, in facilitating ovulation in the rabbit, also lowers various central nervous thresholds to electrical stimulation. There is thus the possibility that treatment of the androgen-sterilized female rat with progesterone may facilitate the electrical stimulus to result in ovulation rather than having a direct effect on pituitary gonadotropin content. Regardless of its site of action, progesterone priming is necessary, prior to hypothalamic stimulation, for ovulation to occur.

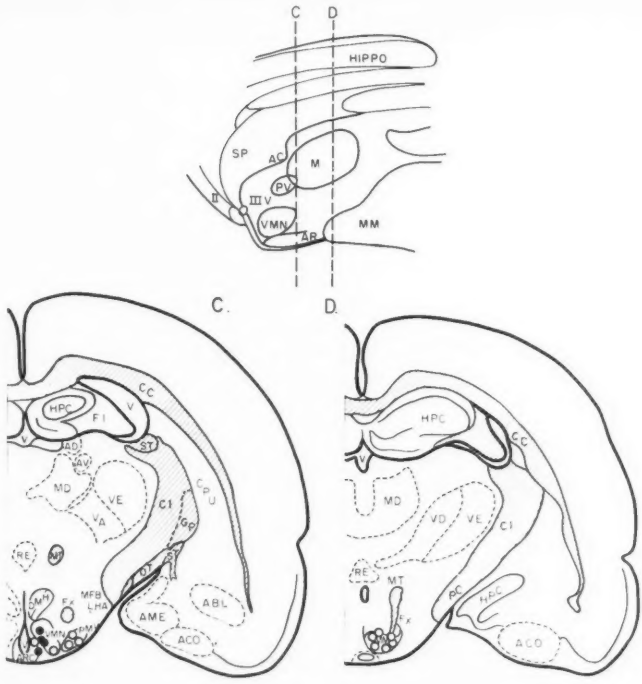

FIG. 6.

FIGS. 5, 6. Midsagittal and coronal sections of rat hypothalamus indicating points of stimulation. Diagrams from DeGroot's "The rat forebrain in stereotaxic coordinates" (15). *Closed circles* indicate hypothalamic sites in which stimulation induced ovulation. *Open circles* represent areas in which stimulation failed to induce ovulation. Abbreviations used in this and subsequent figures: AC, anterior commissure; ACB, area parolfactoria lateralis; AR, arcuate nucleus; CC, corpus callosum; CPU, nucleus caudatus/putamen; CO, optic chiasm; DMH, dorsal medial nucleus; MN, medial mamillary nucleus; MT, mamillothalamic tract; POA, preoptic area; VMN, ventral medial nucleus.

What is the mechanism by which androgen induces sterility? Apparently prepubertal androgen treatment so alters normal hypothalamic function as to render it incapable of activating the cyclic ovulatory discharge of gonadotropin (presumably LH). In contrast, such treatment fails to cause complete cessation of LH secretion. This is evidenced by the syndrome which ensues following prepubertal treatment: persistent vaginal cornification and ovarian interstitial cell, adrenal and pituitary hypertrophy which are either directly or indirectly (through estrogen secretion), the consequence of a tonic discharge of adenohypophyseal LH.

To establish a hypothalamic locus which is deleteriously affected by

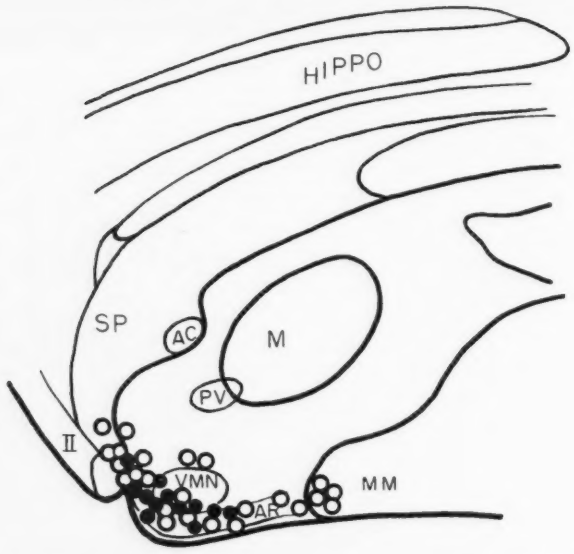

FIG. 7. Midsagittal reconstruction of rat hypothalamus indicating extent of area in which stimulation resulted in ovulation. Abbreviations: hippo, hippocampus; M, massa intermedia; PV, paraventricular nucleus; SP, septum; II, optic nerve.

androgen requires an evaluation of the specific hypothalamic areas proposed to be responsible for the control of ovulation in the normal rat. Critchlow initially demonstrated that electrical stimulation of hypothalamic regions extending from the basal area, from the optic chiasm to the infundibular stalk, consistently induced ovulation in Nembutal-blocked rats (7). The more recent studies of Everett have extended these observations to include the preoptic area rostral to the suprachiasmatic nucleus (8). Furthermore, when small, specific lesions are made in the suprachiasmatic nucleus, reproductive behavior is so affected so as to result in anovulatory-persistent-estrous animals similar in every respect to the androgen-sterilized rat (12).

The hypothalamic locus thus implicated as the site of deleterious androgen action is the midline suprachiasmatic-preoptic area. Lesions of this region result in an imbalance in gonadotropin secretion which imitates that observed in the sterilized rat (12) and ovulation can readily be induced by stimulation of this region in normal but not in androgen-sterilized animals.

The observations of these and previous investigations suggest a dual hypothalamic control of adenohypophyseal gonadotropin secretion in the female rat. The first level of hypothalamic control involves the tonic discharge of gonadotropin in sufficient quantity to maintain estrogen production but cannot independently initiate the ovulatory surge of gonadotropin.

This control is apparently resident in the arcuate-ventromedial nuclei region of the median eminence. Evidence for this primary control and localization is based on the observations that estrogen is secreted in the anovulatory-persistent estrous rat and that electrical stimulation of these structures in the sterile rat will induce LH secretion. Furthermore, destruction of these areas results in the cessation of estrogen production, ovarian atrophy and anestrus (12).

Of fundamental importance is the second and higher control which results in the cyclic discharge of gonadotropin to cause ovulation. The specific region responsible for such control may, most likely, be placed in the preoptic area of the hypothalamus. Furthermore, this region of "ovulation control" is dependent for its activation on exteroceptive (light, etc.) (13), and interoceptive (steroid [14], higher neural control) influences.

Thus it may be that the hypothalamic events which occur during the normal cycle in the female rat are these: the preoptic (suprachiasmatic) area responds under proper environmental and hormonal circumstances,

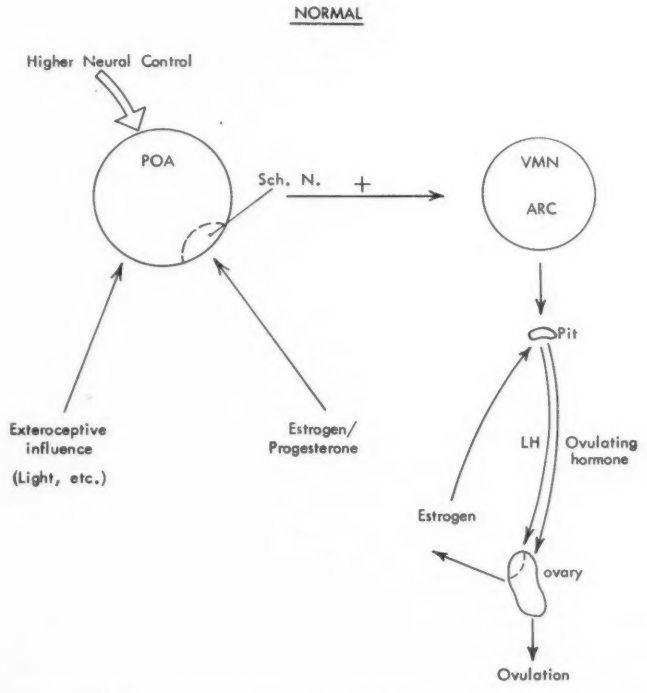

FIG. 8. Diagrammatic representation of hypothalamic events which regulate the discharge of gonadotropin to cause ovulation (ovulating hormone) and/or the tonic release of ovarian estrogen. Abbreviations: POA, preoptic area; Sch. N., suprachiasmatic nucleus; VMN, ventral medial nucleus; ARC, arcuate nucleus.

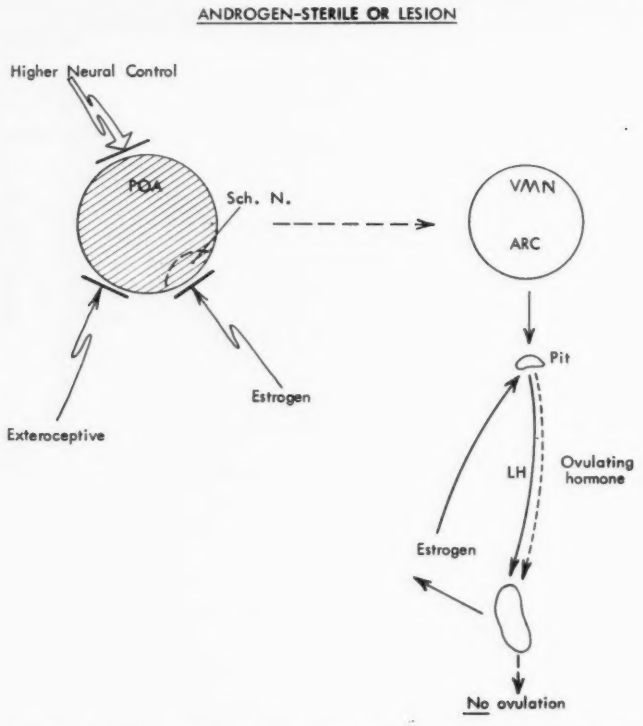

FIG. 9. Hypothalamic events which occur on prepubertal treatment of female rats with androgen or destruction of the anterior preoptic area.

(which are fulfilled on the day of proestrus) by an activation of the more terminal infundibular regions to cause an ovulatory discharge of gonadotropin from the adenohypophysis (Fig. 8). In the absence of this higher control, the terminal structures (arcuate-ventromedial nuclei) still function normally to stimulate LH secretion, but the ovulatory surge of gonadotropin is absent and sterility ensues (Fig. 9).

Seemingly, the anterior preoptic area of the prepubertal female rat is undifferentiated at birth with regard to its subsequent control of gonadotropin secretion. When allowed to differentiate normally it regulates the release of ovulating hormone. However, if differentiated in the presence of androgen, this area becomes refractory to both intrinsic and extrinsic activation and the more tonic type of male gonadotropin secretion is observed.

Acknowledgments

The authors wish to express their appreciation for the technical assistance rendered by Miss Theresa Mangold and Mr. Leroy Brown during this study.

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MAGNESIUM DEFICIENCY IN INTACT, IN ADRENALECTOMIZED AND IN HYPOPHYSECTOMIZED RATS[1]

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ABSTRACT

Control and magnesium-deficient synthetic diets were fed *ad libitum* to intact, to adrenalectomized and to hypophysectomized male rats for 8, 15 and 22 days. The course and sequence of the appearance of magnesium-deficiency symptoms were recorded. In the intact and the adrenalectomized rats fed the deficient diet, the growth rate was depressed, whereas the hypophysectomized deficient rats showed no significant difference in weight gain compared to their controls.

Rats in each of the three groups on the deficient diet developed hyperemia, leucocytosis (especially eosinophilia) and renal lesions in the distal convoluted tubules coincident with the initial depletion of serum magnesium. In the intact and the adrenalectomized rats these magnesium-deficiency symptoms appeared during the first week on the experimental diet, but in the hypophysectomized rats the onset of the symptoms was significantly delayed to the third week.

Although the kidney lesions were similar to those described for the potassium-deficient rats, they appeared to be due to magnesium deficiency *per se* in these animals. The data also suggested a possible pituitary-kidney axis for the control of serum magnesium level.

INTRODUCTION

IT IS well known that hormones actively participate in the body's metabolism of inorganic ions, such as the effects of parathyroid hormone on calcium and aldosterone on sodium and potassium. The belief that the serum magnesium, like calcium, sodium and potassium, may be actively controlled by hormones is supported by observations on the fluctuation of this cation in certain clinical diseases such as primary aldosteronism (1, 2) and Addison's disease (3), and in certain physiological states such as hibernation (4, 5), sleep (6) and low environmental temperature (7, 8, 9). Reports on the conservation of magnesium by the kidneys during a deficiency state (10, 11) provide further evidence of active control of this cation. Hormonal control of magnesium has been suggested in

Received June 13, 1960.

[1] The data in this paper were presented as part of a dissertation to the Graduate Council of The George Washington University in candidacy for the degree of Doctor of Philosophy.

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studies showing fluctuation of serum magnesium level after removal of certain endocrine glands, associated with hyperactive glands or after the injection of various hormones. However, these reports confuse the problem by the implication of several hormones, namely thyroxine (12, 13), parathyroid hormone (14, 15, 16), ACTH (17) and aldosterone (1, 2). It is highly improbable that all of these hormones are involved in controlling the single cation, magnesium.

In view of the uncertainties in our knowledge of magnesium control, an attempt was made in this investigation to re-evaluate the effects of adrenalectomy and of hypophysectomy on magnesium metabolism (18). The sequential appearance of some of the known magnesium-deficiency symptoms (19) and the recently described leucocytosis (20) was correlated with the depletion of serum magnesium in intact, in adrenalectomized and in hypophysectomized rats.

MATERIALS AND METHODS

Control and magnesium-deficient diets containing 0.72% calcium and with a Ca:P ratio of 1.25 were prepared and fed *ad libitum* to intact, to adrenalectomized and to hypophysectomized rats for 8, 15 and 22 days. The control diet contained 34 mg.% and deficient diet contained 0.37 mg.% magnesium. The composition of these diets has been described elsewhere (20).

TABLE 1. EXPERIMENTAL DESIGN: DISTRIBUTION OF INTACT, OF ADRENALECTOMIZED AND OF HYPOPHYSECTOMIZED RATS FED CONTROL AND MAGNESIUM-DEFICIENT DIETS FOR 8, 15 AND 22 DAYS

Group	Days on diet	Diet	No. of rats		
			Intact	Adrenx.*	Hypx.†
1	0‡	Purina lab. chow	8	8	10
2	8	Control	7	8	10
3	8	Deficient	10	8	10
4	15	Control	7	8	10
5	15	Deficient	10	8	10
6	22	Control	20	10	10
7	22	Deficient	25	10	13

* Adrenalectomized.
† Hypophysectomized.
‡ For convenience, rats sacrificed prior to the feeding of the synthetic diets will be called Day 0 group of rats.

Eighty-seven intact, male Wistar rats weighing about 80 gm. were distributed at random to comprise seven groups as shown in Table 1. During the experimental feeding all rats in this group received demineralized drinking water.

Sixty male Wistar rats weighing about 80 gm. were bilaterally adrenalectomized through the dorsal approach. After the fifth postoperative day they were distributed at random into seven groups as shown in Table 1. During the period of experimental feeding the animals were maintained on 1% NaCl prepared with demineralized water.

Seventy-three hypophysectomized male rats of the Sprague-Dawley strain, weighing about 100 gm., were purchased from the Hormone Assay Laboratories, Inc. and were distributed into seven groups as shown in Table 1. During the experiment the drinking solution, 5% glucose and 0.5% NaCl, was prepared with demineralized water.

Body weights were recorded a day before the feeding of the synthetic diets (Day 0) and on days 7, 14 and 21. The hyperemic ears were scored from 1+ to 4+ using the indices previously described (20). The daily hyperemic indices of a group were converted to percentile figures by dividing the total daily indices by the maximum hyperemia possible for the group (4+ times total number of rats) and multiplied by 100. The total WBC, direct eosinophil counts and differential counts of neutrophils and mononuclear leucocytes were made on days 0, 8 and 15 only in those rats that were fed the diet for 15 and 22 days. The methods used have been described elsewhere (20).

Blood samples were taken by heart punctures in heparinized syringes and the total serum magnesium determined by the titan yellow method as recommended by Orange and Rhein (21). Pituitaries were fixed in Bouin's solution, dehydrated, cleared and embedded in paraffin by the standard procedure. The sections were stained with chrome alum hematoxylin for neurosecretory substances (22) and adjacent sections were stained with the aldehyde fuchsin method for cell types in the anterior lobe (23). Adrenal glands were fixed in 10% formalin and frozen sections were cut and stained with oil red 0 for lipids (24). The kidneys were fixed in 10% formalin and processed through paraffin in the usual manner. Alternate sections were stained with hematoxylin and eosin, von Kossa's method for calcium (24) and periodic acid-Schiff stain (23).

RESULTS

Body Weight

The growth rates of the intact and the adrenalectomized rats fed the magnesium-deficient diet were less than those of their respective control rats (Table 2). The body weights of both these deficient groups were significantly lighter ($P < .01$) than their controls after the first week on diet. On day 21 the weights of the intact and the adrenalectomized deficient rats were 70% of that of the control animals. The control rats grew nor-

TABLE 2. AVERAGE BODY WEIGHTS OF INTACT, OF ADRENALECTOMIZED AND OF HYPOPHYSECTOMIZED RATS FED CONTROL AND MAGNESIUM-DEFICIENT DIETS

Group	No. of rats	Days on diet			
		0	7	14	21
Intact—C	20	88 ± 11*	120 ± 13	146 ± 17	165 ± 22
Intact—D	17	87 ± 12	111 ± 14	115 ± 17	116 ± 18
Adrenx.—C	7	91 ± 6	126 ± 11	167 ± 21	185 ± 21
Adrenx.—D	9	99 ± 10	118 ± 10	124 ± 10	131 ± 13
Hypx.—C	10	102 ± 4	98 ± 5	91 ± 10	99 ± 8 (9)†
Hypx.—D	13	100 ± 7	99 ± 7	92 ± 6	92 ± 6 (7)

* Standard Deviation.
† No. of rats alive on day 22 in parentheses.

mally and seemed to be in good health. The mortality rates for rats on the diet for 22 days were 32% for the intact deficient group, 10% for the adrenalectomized deficient group and 30% for the adrenalectomized control group. None of the intact control rats died.

In the hypophysectomized group the growth rates for the control and the magnesium-deficient rats paralleled each other, both showing a slight loss in body weight throughout the experiment (Table 2). On day 21 the control group was 97% and the deficient group 92% of the initial body weight. The largest difference (7 gm. on day 21) between the body weights of the control and the deficient hypophysectomized rats was statistically insignificant ($P > .05$). The mortality rate for the hypophysectomized rats on the diet for 22 days was 46% for the deficient group and 10% for the control group.

Hyperemia

The intact as well as adrenalectomized and the hypophysectomized rats fed the magnesium-deficient diet developed hyperemic ears while none of the control rats exhibited this symptom. In the intact rats the hyperemia

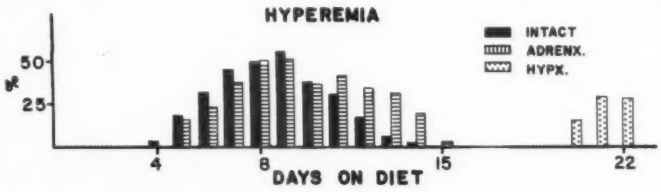

FIG. 1. Hyperemia in intact, adrenalectomized and hypophysectomized rats fed magnesium-deficient diet for 22 days.

appeared on the 4th day, reached its peak on the 9th day and blanched by the 15th day (Fig. 1). In the adrenalectomized deficient group the initial appearance and final blanching of the symptom occurred a day later than in the intact deficient group. This difference was statistically insignificant ($P > .05$). In contrast, the appearance of hyperemic ears in the hypophysectomized deficient group was delayed until the 20th day, a delay of about 16 days compared to the intact and adrenalectomized deficient groups. The average duration of hyperemia was 11 days in the intact and the adrenalectomized deficient groups. The duration could not be determined for the hypophysectomized deficient rats since the experiment was terminated two days after the first appearance of this symptom.

The individual variation of hyperemic response is outlined in Table 3. Not all rats placed on the magnesium-deficient diet developed this vascular reaction. In the intact group 92% of the rats developed hyperemia, whereas all of the 24 adrenalectomized rats examined exhibited this response. Out of the 7 hypophysectomized rats which survived to day 22, only two rats

TABLE 3. HYPEREMIA IN INTACT, IN ADRENALECTOMIZED AND IN HYPOPHYSECTOMIZED RATS FED MAGNESIUM-DEFICIENT DIET

	No. of rats	% of rats developing hyperemia	Duration of hyperemia per rat (days)	
			Average	Range
Intact	24	92	5	2–8
Adrenx.	18	100	5	3–8
Hypx.	7	29*	Insufficient Data	

* Not representative since rats in this group were sacrificed 2 days after the first onset of hyperemia.

became hyperemic. The onset of hyperemia differed from rat to rat; the vascular reaction developed on day 4 in some rats and on days 5, 6 or 7 in others. The severity of hyperemia also varied; in some rats the hyperemia progressed to the 2+ stage and blanched without further development while in others the markedly congested blood vessels of the ears (4+) persisted for several days. The duration of hyperemia per intact or adrenalectomized rat averaged 5 days with a range from 2 to 8 days.

White Blood Cell Counts

The average WBC counts of the intact control group increased gradually during the three weeks on the synthetic diet (Table 4). In the intact group fed the deficient diet, the total WBC count was markedly elevated to a peak of 30,000 cells per mm.^{3} on day 8 and returned toward the control value on the 15th day. This leucocytosis on day 8 was significant ($P < .01$) when compared to the control level and was due to an increase in the number of mononuclear leucocytes, neutrophils and especially eosinophils in the order of 2.2, 4.5 and 7.5 times the initial level, respectively.

In the adrenalectomized control rats the counts for each cell type and the total WBC did not differ significantly from the initial level (Table 4). In the deficient group, however, all counts increased significantly on day 8 and further on day 15 ($P < .01$). The increase in counts from the prediet

TABLE 4. WHITE BLOOD CELL COUNTS OF INTACT, OF ADRENALECTOMIZED AND OF HYPOPHYSECTOMIZED RATS FED CONTROL AND MAGNESIUM-DEFICIENT DIETS

Group	No. of rats	Total WBC/mm.^{3} (days)			Mononuclear/mm.^{3} (days)			Neutrophils/mm.^{3} (days)			Eosinophils/mm.^{3} (days)		
		0	8	15	0	8	15	0	8	15	0	8	15
Intact—C	15	11.8*	15.5*	24.2*	9.9*	12.4*	18.8*	1.8*	3.0*	3.1*	94	147	314
Intact—D	12	11.9	30.6	26.6	10.0	22.2	19.7	1.8	8.1	6.8	81	611	366
Adrenx.—C	7	14.0	14.9	13.5	11.0	10.5	11.2	2.9	3.0	2.2	195	191	184
Adrenx.—D	10	15.6	27.3	37.6	13.2	21.6	28.4	2.4	5.6	8.6	155	468	1250
Hypx.—C	17	28.2	28.7	28.2	24.6	25.8	25.8	3.3	2.6	2.3	392	373	195
Hypx.—D	17	29.4	36.3	33.3	25.9	31.7	30.8	3.1	4.1	2.4	269	284	163
		Day 22			*Day 22*			*Day 22*			*Day*		
Hypx.—C	9	23.4			20.8			2.5			145		
Hypx.—D	7	47.1			42.7			3.5			921		

* Cells $\times 10^3$.

levels of mononuclear cells, neutrophils and eosinophils were 2.4, 2.3 and 3.0 times respectively on day 8, and 2.1, 3.6 and 8.1 times respectively on day 15.

In the hypophysectomized control rats the total number of WBC and mononuclear cells remained relatively unchanged during the first two weeks but decreased significantly on the 22nd day (Table 4). The neutrophil and eosinophil counts decreased from day 0 to day 22 by a factor of 0.75 and 0.36 respectively. In the deficient group a slight rise in total WBC, mononuclear, neutrophil and eosinophil counts was seen on the 8th day. The most significant change in counts, however, occurred on the 22nd day when all except the neutrophil count were significantly elevated ($P < .01$). The total WBC count was elevated 1.6 times, the mononuclear 1.6 times and the eosinophil 3.4 times the prediet levels.

TABLE 5. SERUM MAGNESIUM LEVELS (MG.%) OF INTACT, OF ADRENALECTOMIZED AND OF HYPOPHYSECTOMIZED RATS FED CONTROL AND MAGNESIUM-DEFICIENT DIETS

Group	Days on diet			
	0	8	15	22
Intact—C	2.51 ± 0.47* (8)†	2.15 ± 0.36 (7)	1.97 ± 0.18 (7)	1.67 ± 0.28 (13)
Intact—D		0.38 ± 0.25 (10)	0.34 ± 0.17 (9)	0.57 ± 0.53 (8)
Adrenx.—C	2.22 ± 0.11 (6)	1.61 ± 0.17 (6)	1.86 ± 0.32 (6)	2.09 ± 0.21 (7)
Adrenx.—D		0.39 ± 0.23 (6)	0.80 ± 0.48 (7)	0.94 ± 0.60 (8)
Hypx.—C	2.09 ± 0.30 (8)	2.20 ± 0.66 (10)	2.48 ± 0.70 (10)	2.15 ± 0.29 (8)
Hypx.—D		2.10 ± 0.58 (8)	1.04 ± 0.42 (8)	0.51 ± 0.29 (6)

* Standard Deviation.
† No. of rats in parentheses.

A period of general leucocytosis was observed in each group of rats fed the magnesium-deficient diet. A significant rise occurred on the 8th day in the intact and the adrenalectomized groups and on the 22nd day in the hypophysectomized group. The leucocytosis was due in each case to the elevation of all three white blood cell types: mononuclear cells, neutrophils (except in the hypophysectomized deficient group) and eosinophils. In each group, however, the greatest increase from the prediet level was seen in the eosinophil count.

Serum Magnesium

The serum magnesium levels of the control rats of the three groups (Table 5) remained within the normal range. In the intact rats fed the magnesium-deficient diet, the average total serum magnesium levels (Table 5) dropped from a prediet level of 2.51 mg.% to a low value of 0.38 mg.% on the 8th day and remained low throughout the remainder of the experiment. The serum magnesium level of the adrenalectomized deficient rats (Table 5) similarly dropped to a low value of 0.39 mg.% on day 8 from

a prediet level of 2.22 mg.% but thereafter began to rise, attaining a value of 0.94 mg.% on the 22nd day ($P = .05$). The serum magnesium level of the hypophysectomized deficient rats (Table 5) maintained the prediet level of serum magnesium on day 8, but began to decline on day 15, reaching a value of 1.04 mg.%, a significant drop from the day 8 value and from their controls ($P < .01$). The 22nd day value reached a low of 0.51 mg.% magnesium.

Histological Examination

The concentration and distribution of oil red O-positive lipids in the adrenal cortex; the neurosecretory substances of the posterior pituitary lobe stained with chrome alum hematoxylin; and the cytology and number of alpha and beta cells of the anterior pituitary lobe stained with PAS or aldehyde fuchsin showed no significant differences between the control and magnesium-deficient rats.

Kidney

The kidneys of intact, of adrenalectomized and of hypophysectomized deficient rats developed tubular lesions in the cortex (Figs. 2 and 3) and at the cortico-medullary junction (Figs. 4 and 5). In the intact and adrenalectomized deficient rats these lesions appeared as early as day 8 and persisted throughout the duration of the experiment. The kidneys of the hypophysectomized rats, fed the deficient diet for 8 and 15 days, appeared normal but tubular lesions appeared in kidneys of hypophysectomized rats fed the deficient diet for 22 days.

The histologic changes in the kidneys were as follows:

1. Foci of tubules in the cortex lined by hypertrophied cells with enlarged, vesiculated nuclei and vacuolated cytoplasm (Fig. 2).
2. Foci of tubules in the cortex lined by flattened epithelial cells with enlarged lumina (Fig. 3).
3. Tubules lined by cells with pyknotic nuclei and eosinophilic cytoplasm, most numerous at the cortico-medullary junction (Fig. 4).
4. Foci of basophilic, von Kossa-positive, calcareous deposits in tubules, especially numerous at the cortico-medullary junction (Fig. 5).

Certain lesions seemed to be more predominant during the early stage and others during the late stage of deficiency. In kidneys of the intact and the adrenalectomized rats fed the deficient diet for 8 and 15 days, lesions 1 (hypertrophied cells), 2 (flattened epithelium) and especially lesion 3 (pyknotic nuclei) were more numerous than lesion 4 (calcium deposits). On the 22nd day, lesion 4 was the primary one in the intact and the adrenalectomized rats. In the hypophysectomized deficient rats on day 22, lesion 1 was most predominant with the appearance of occasional lesions 2, 3 and 4.

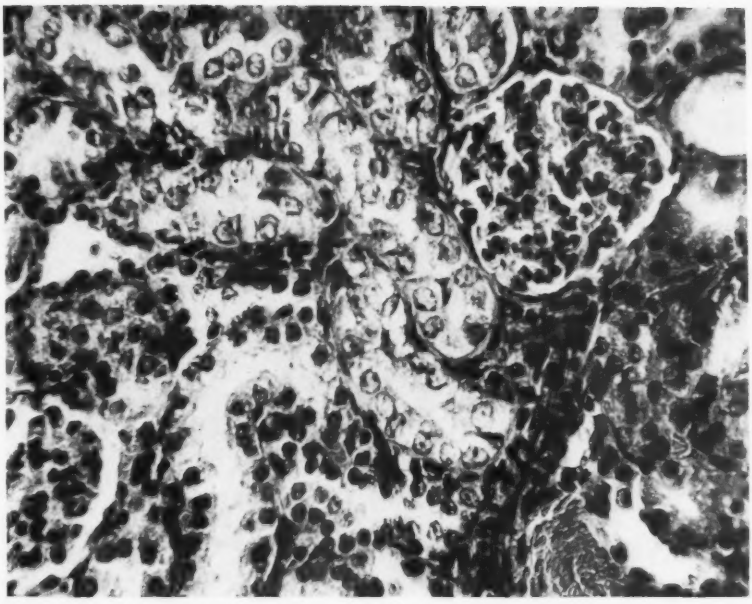

FIG. 2. Cortex of kidney from a hypophysectomized rat fed magnesium-deficient diet for 22 days, showing tubules lined with hypertrophied cells. Hematoxylin and Eosin. 320×

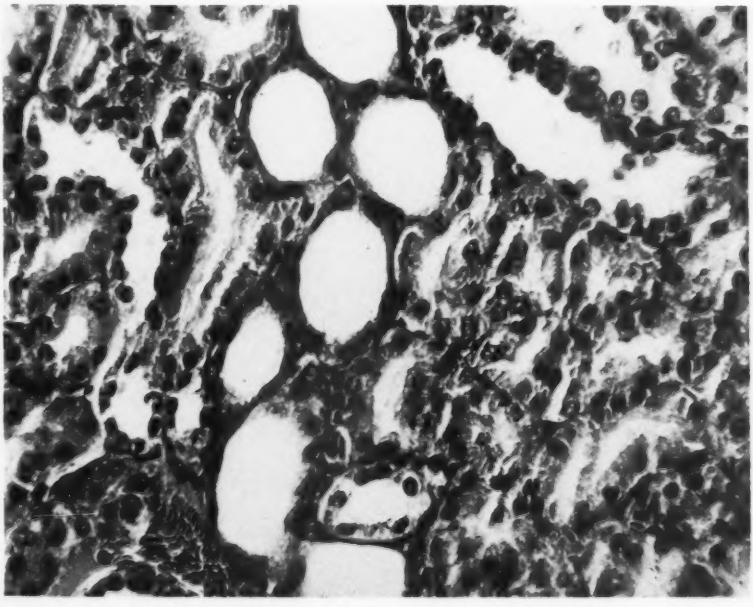

FIG. 3. Cortex of kidney from a hypophysectomized rat fed magnesium-deficient diet for 22 days, showing tubules lined with flattened epithelium. Hematoxylin and Eosin. 320×

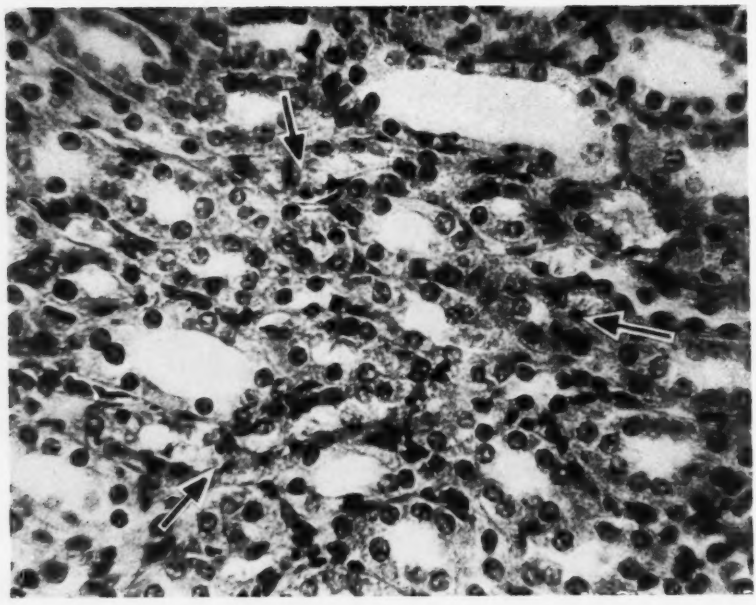

FIG. 4. Cortico-medullary junction of kidney from an adrenalectomized rat fed magnesium-deficient diet for 15 days, showing tubular cells (arrows) with pyknotic nuclei and eosinophilic cytoplasm. Hematoxylin and Eosin. 320×

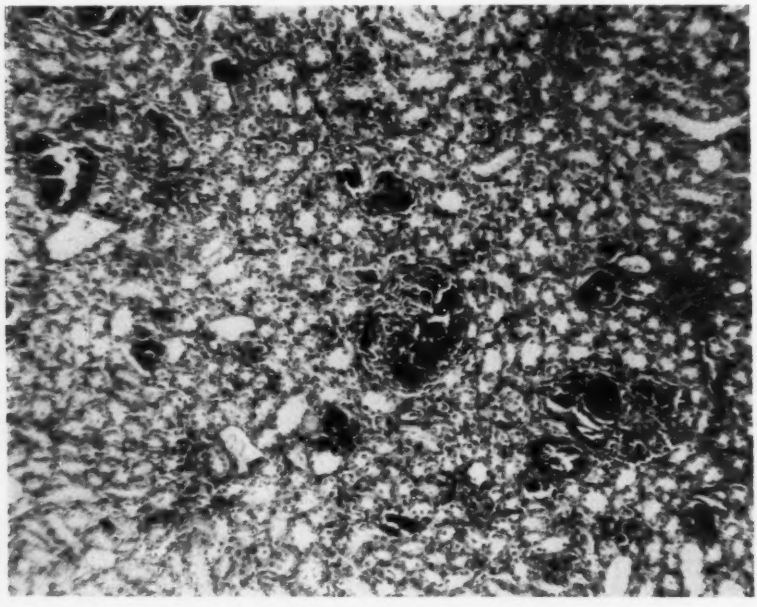

FIG. 5. Cortico-medullary junction of kidney from an intact rat fed magnesium-deficient diet for 22 days, showing calcium deposition within tubules. Hematoxylin and Eosin. 170×

DISCUSSION

The primary concern of this discussion shall be to elaborate on the observation that adrenalectomized rats developed magnesium-deficiency symptoms coincident with the intact rats, whereas the deficiency symptoms were definitely delayed in the hypophysectomized rats. This delayed depletion of serum magnesium and the consequent appearance of symptoms in the hypophysectomized animals further substantiates the symptoms as being truly due to magnesium deficiency and suggests an interesting relationship between the pituitary and magnesium metabolism.

In groups of intact as well as adrenalectomized rats, we were able to confirm the observations made by Kruse, Orent and McCollum (19) and by Tufts and Greenberg (25) that hyperemia appears on the 4th day, reaches its peak on about the 8th to 9th day and blanches on the 14th day. The correlation between the initial decrease in serum magnesium level and the appearance of hyperemia is also in agreement with the concept proposed by Bélanger *et al.* (26) that the hyperemia is initiated by histamine liberated from mast cells due to stimulation by low serum magnesium. However, we were not able to confirm the proposed explanation by Bélanger *et al.* that the hyperemia blanches because of a rise in serum magnesium level (25) on day 15. During the period of blanching of the hyperemia on day 15, the serum magnesium level in our intact deficient rats was as low as the value for day 8 when hyperemia was most severe.

The direct cause or reason for leucocytosis and renal lesions in the magnesium-deficient rats is not known but that their appearance is coincident with hyperemia and serum magnesium depletion is established by our data. Perhaps our contention that the drop in serum magnesium level initiates hyperemia, leucocytosis and renal pathology is best substantiated by our observations on hypophysectomized rats. In this group the symptoms did not appear on days 8 or 15, as in the intact and adrenalectomized rats, but on the 22nd day when the serum magnesium level was finally depleted to 0.5 mg.%.

The delay in the depletion of serum magnesium and the appearance of symptoms in the hypophysectomized rats suggests that the pituitary may be involved either directly or indirectly in the control of serum magnesium; in the absence of pituitary the normal serum magnesium level persisted in spite of low magnesium consumption. Magnesium, injected intravenously, has been shown to be excreted mainly by the kidney (27, 28). The kidney is known to conserve magnesium (10, 11) and it probably does so by the reabsorption of this cation in the distal convoluted tubules (29). In view of these reported observations and the present data, it would seem that there may be a pituitary-kidney axis which controls magnesium excretion. However, our examination of pituitaries from intact and adrenalectomized deficient rats did not reveal changes in the cytology of anterior lobe or in the concentration of neurosecretory substance to indicate activity which differed from their respective controls.

Although the reason for the delay in the depletion of serum magnesium in the hypophysectomized rats is not known, we can infer from the data on adrenalectomized rats that the absence of ACTH was not the factor responsible since the adrenalectomized rats did not deviate from the time sequence established for the intact rats. The histological studies of the adrenals of the intact and of the hypophysectomized rats indicated activity commensurate to that of the respective controls and no sign of activity due to magnesium deficiency could be seen. Furthermore, recent reports have denied the existence of control of serum magnesium levels by adrenal cortical hormones (30–33).

The renal lesions, especially the hypertrophied tubular cells and tubules with flattened epithelial cells, were similar to those described in potassium-deficient rats (34). In the kidney of magnesium-deficient rats, however, deposition of fat in the cytoplasm of renal tubular cells, described in potassium-deficient rats, was not found. In the potassium-deficient rats, calcium deposition in the renal tubules is not as intensive as it is in the magnesium-deficient rats. We believe that the renal cortical lesions in our deficient rats involved the distal convoluted tubules or the distal portion of the nephron since brush borders were absent from these hypertrophied cells and since the area around the lesion in the cortex was made up of many histologically normal proximal convoluted tubules with distinct brush borders and eosinophilic cytoplasm. In the potassium-deficient rats the lesions were described to be in the proximal portion of the nephron.

Acknowledgments

The author wishes to thank Drs. Ira R. Telford and Gerald F. Hungerford for their valuable assistance during the course of the investigation.

This investigation was supported in part by grant from the Washington Heart Association, Washington, D. C. and completed during the tenure of a Predoctoral Research Fellowship of the National Heart Institute, National Institutes of Health, Bethesda, Maryland.

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ENDOCRINE REGULATION OF OVIDUCT SECRETIONS IN THE RABBIT[1]

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ABSTRACT

A system for prolonged volumetric collection of oviduct secretions was applied to adult New Zealand White rabbits. The effect on oviduct secretion rate of castration, progesterone, and postcastration estrogen and/or progesterone treatment was evaluated. After castration, the rate of oviduct secretion fell, reaching a plateau at about one-third the precastration level within 24–124 hours (mean 67 hours). The secretion rate of the castrate increased following estrogen administration. This effect was obtained with doses as low as 0.5μg. By continuous daily administration of estrogen, the oviduct secretion rate could be increased to precastration levels. Control injections of sesame oil failed to produce a change. In the intact animal, a single injection of 3 mg. of crystalline progesterone in 0.1 ml. of sesame oil was followed by a decrease in secretion rate which was most pronounced on the second posttreatment day. The oviduct secretion rate was uniformly depressed in the estrogen primed castrate (2 μg. of estradiol 17β daily) by administration of 1 mg. of progesterone in 1 ml. of sesame oil twice daily for three days. The rate became lowest on the third day of treatment. Castrates receiving a similar dosage schedule of progesterone in the absence of estrogen failed to exhibit a decrease. The data presented justify the conclusion that the rate of secretion in the rabbit oviduct is under endocrine control.

HORMONE regulated alterations in the secretory cells of the oviduct have been observed in several species. Changes during the menstrual cycle of man were emphasized in 1928 by Novak and Everett (1). They described a heightening of the epithelium in the "interval" phase of the cycle. Premenstrually the ciliated cells became lower, and the secretory cells projected beyond them. More recently, Fredricsson (2),

Received June 15, 1960.

[1] This investigation was supported in part by a grant from the Josiah Macy, Jr. Foundation and by P. H. S. Grant #RG 5344 from the National Institutes of Health, United States Public Health Service.

The authors are grateful to Mrs. Marianne Walker for technical assistance, and to Mrs. Julia Zalicar, Department of Public Health, for statistical treatment of the data. Estradiol and progesterone were generously supplied by the research division of Schering Inc.

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using histochemical techniques, found secretory development to be most marked in the midluteal phase of the cycle, with some increase in intraluminal secretions at ovulation. The alterations in the appearance of the secretory cells of the rabbit oviduct have been studied with the electron microscope by Borell, *et al.* (3). Typical secretory cells were not observed in the castrate, but some cells containing solitary secretory granules were visible. In anestrus, the secretory cells were flush with the ciliated cells and contained only a few granules. During estrus, the secretory cells bulged into the tubal lumen and contained dense granules. These cellular modifications suggest that the secretory process in the oviduct is under endocrine control.

Attention was focused on the oviduct secretions themselves in 1891 by Woskressenski (4). He observed an accumulation of fluid in the rabbit oviduct following ligation at the uterotubal junction and the fimbriated extremity. The fluid contained between the ligatures was sufficient to distend the oviduct. D. W. Bishop (5) has measured the rate of fluid accumulation in rabbit oviducts ligated at each end. Fluid production was slower in the castrate and in the pregnant animal than in the estrous subject. Using a manometric technique in anesthetized rabbits, Bishop (6) demonstrated that the oviduct fluid was secreted against a pressure gradient. Secretion pressure rose following administration of pilocarpine. He concluded that the fluid represented an active secretion and not a transudate. Zachariae (7), using autoradiography, demonstrated an active deposition of S^{35} into the rabbit oviduct lumen in the form of acid sulphuric mucopolysaccharide. He compared the secretory process of the oviduct to that of other mucopolysaccharide producing glands. Greenwald (8) has shown that the secretion of mucin by the rabbit oviduct can be modified by exogenous hormones. Estrogen reduced deposition of mucin into the oviduct lumen; progesterone increased it.

The present study was designed to evaluate the influence of progesterone, castration, and postcastration estrogen and/or progesterone on the oviduct secretion rate in unanesthetized rabbits. A system for prolonged, volumetric collection of oviduct secretions was employed. A detailed description of the collecting system, the principles underlying its use and preliminary experiences with it have been presented in an earlier publication (9).

MATERIALS AND METHOD

To collect oviduct fluid and measure its secretion rate, a volumetric collecting system was applied to New Zealand White rabbits weighing 3–5 Kg. The abdomen was entered, and an oviduct was ligated and transected at the uterotubal junction. A silastic (T. M. Reg. Dow Corning Corp.) cannula, connected to a calibrated collecting chamber was led through a lateral abdominal stab wound. The collecting chamber was secured to the external body wall in a midlateral position. The tip of the cannula was secured just within the tubal ostium with a fine silk suture. The collecting apparatus consisted of a siliconized glass lumen imbedded in clear plastic. The lumen was fashioned in a zig-zag pat-

tern and calibrated at 0.01 ml. increments. It was vented to the atmosphere. A section of India rubber tubing, located between the glass lumen and the cannula, permitted withdrawal of accumulated fluid by needle aspiration. The position of the collecting lumen relative to the oviduct resulted in collection of fluid at a minimal pressure gradient. Such a collecting system usually remained functional over many days, permitting evaluation of the effect of changing hormonal conditions on the oviduct secretion rate in a given subject.

The rabbits were housed in separate cages and received a diet of Purina chow *ad libitum*.

RESULTS

The Effect of Castration on Oviduct Secretion Rate. After a steady secretion rate had been observed for several days, bilateral ovariectomy was carried out in 19 animals. The collecting system continued to function postoperatively in 13. In each, the secretion rate decreased following castration, leveling off at a lower plateau within 25–124 hours (mean 64 hours). The secretion rate remained steady at the new plateau for 4 to 5 days. Thereafter, a very gradual recession in rate was observed. The precastration secretion rate for the group averaged 0.06 ml./hr. (median 0.06 ml./hr.). The mean and median postcastration rates, after the initial decrease, were 0.02 ml./hr.

The Estrogen Treated Castrate. Twelve animals which were castrated while the secretion rate was under observation were treated subsequently with single subcutaneous injections of estradiol 17β. Doses ranging between 0.5 and 8 μg. were employed. One-tenth ml. of sesame oil was the vehicle throughout.

Four animals received 8 μg. of estradiol 17β 7 to 9 days after castration (Fig. 1). In each, the secretion rate increased, reaching a maximun within 24 to 48 hours (mean 33 hours) after treatment. The mean precastration rate for the group was 0.06 ml./hr. The postcastration plateau averaged 0.02 ml./hr. The mean maximal response to 8 μg. of estradiol 17β was 0.05 ml./hr. The secretion rate remained unchanged following control injections of sesame oil.

One animal which was observed for 55 days after castration received periodic injections of estradiol 17β in doses ranging from 0.5 to 8.0 μg. (Fig. 2). Each was followed by a temporary increase in the secretion rate. At first glance there appeared to be a diminishing response to decreasing amounts of estrogen. This was paralleled, however, by a gradual decrease in the baseline rate between each injection.

In the group, 6 animals received 8 μg., five 4 μg., three 2 μg., one 1 μg., and two 0.5 μg. of estradiol 17β. After each injection there was an increase in the secretion rate followed by a return to a level at or slightly below the preinjection, "castrate" level. The percentage rise above the pretreatment level was essentially the same for various dosage levels. The mean maximum increase after 8 μg. in 6 animals was 260% $\pm$S.D. 29.5. Among 5 animals treated with 4 μg., it was 255% $\pm$S.D. 32. Among 6 animals re-

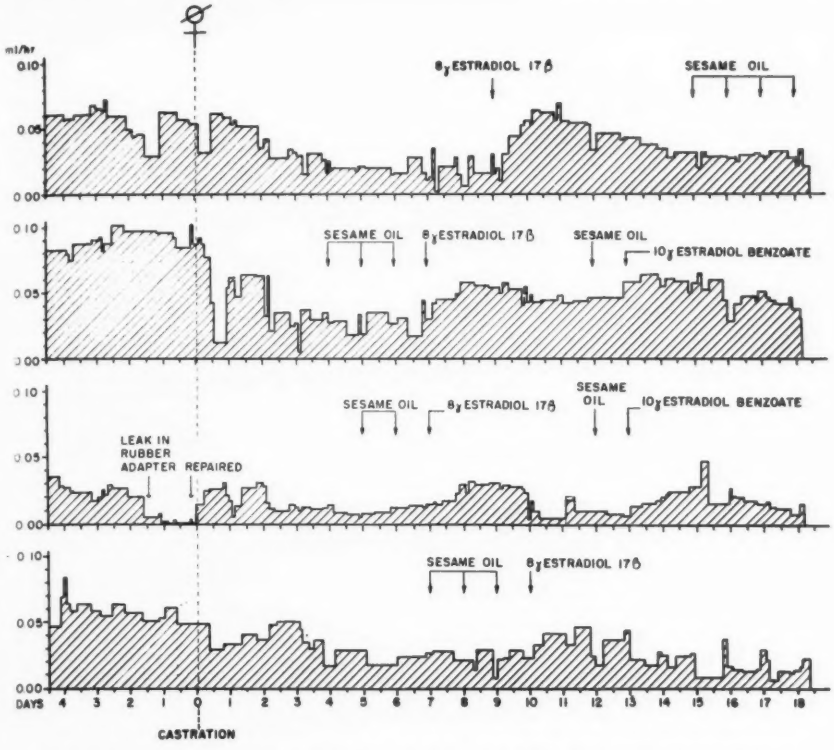

FIG. 1. Oviduct secretion rate (ml./hr.) in four rabbits pre- and postcastration and after a single injection of 8 μg. of estradiol 17β in 0.1 ml. of sesame oil.

ceiving between 0.5 and 2 μg., the mean increase was 290%+S.D. 21. Sporadic control injections of sesame oil were not associated with a change in the secretion rate.

In one animal, after the secretion rate had stabilized at a lower plateau following castration, 5 μg. of estradiol benzoate were administered daily for 6 consecutive days (Fig. 3). The secretion rate rose to the precastration level on the third day of treatment. It was maintained at that level until 4 days after the end of treatment.

Twelve animals were oophorectomized at the time the system was applied. Twenth-four hour secretion rates were recorded. The system remained functional in 9 (Table 1). After the secretion rate had reached a postcastration plateau (24-hour periods 2 and 3), daily subsutaneous injections of 4 μg. of estradiol 17β in 0.1 ml. of sesame oil were administered. In each of 5 subsequent 24-hour periods the secretion rate rose significantly (periods 4–8). The rate reached a maximum plateau on the fifth day of treatment (period 8), where it was maintained (period 9).

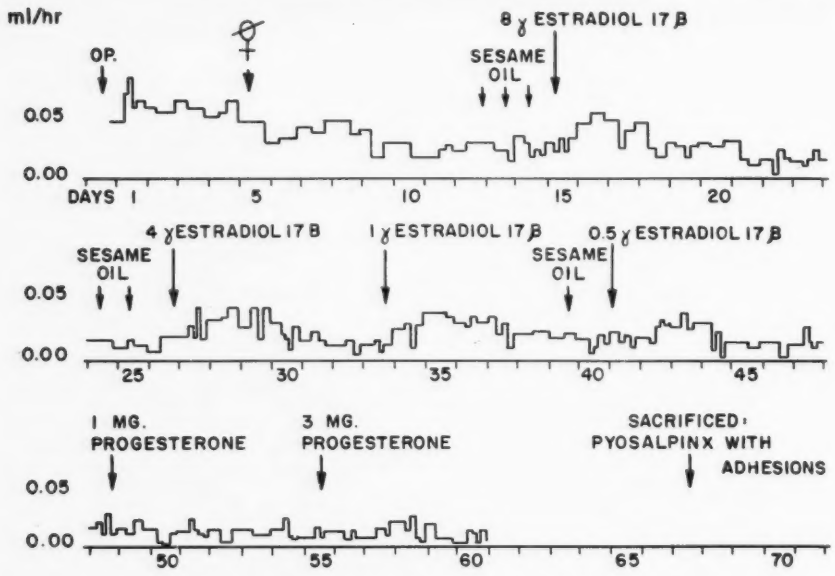

FIG. 2. Oviduct secretion rate (ml./hr.) of an animal which received periodic injections of estradiol 17β in diminishing amounts following castration.

The Progesterone Treated Intact Animal. Nine rabbits with functioning collecting systems received 3 mg. of crystalline progesterone in 0.1 ml. of sesame oil subcutaneously. Twenty-four hour secretion rates were calculated for each (Table 2). The secretion rate fell following treatment. The rate was lowest on the second post treatment day and increased thereafter. The decrease on the second day following treatment was statistically sig-

TABLE 1. OVIDUCT SECRETION RATES (ML./24 HR.) AMONG NINE CASTRATES, BEFORE AND DURING TREATMENT WITH DAILY SUBCUTANEOUS INJECTIONS OF 4 μG. OF ESTRADIOL 17β/0.1 ML. OF SESAME OIL

24 hr. periods	1	2	3	4	4 μg. of estradiol 17β daily				
					5	6	7	8	9
Animal No.									
1	0.28	0.29	0.30	0.72	1.03	1.08	1.09	1.63	1.20
2	0.29	0.27	0.30	0.68	1.16	1.32	1.40	1.43	1.28
3	0.18	0.20	0.17	0.35	0.94	1.16	1.35	1.26	0.94
4	0.60	0.50	0.51	0.65	1.22	1.51	1.53	1.42	1.46
5	0.42	0.40	0.39	0.45	0.84	1.80	1.89	1.87	1.80
6	0.37	0.32	0.23	0.44	0.53	0.65	0.80	1.06	1.18
7	0.40	0.27	0.21	0.44	0.88	1.22	1.26	1.45	1.45
8	0.32	0.30	0.32	0.39	0.92	1.44	1.94	2.15	2.06
9	0.63	0.68	0.41	0.57	1.24	1.53	1.56	1.83	2.02
Mean	0.39	0.36	0.32	0.52	0.97	1.30	1.42	1.57	1.49

For P = 0.05, the just significant difference between two means is 0.10.

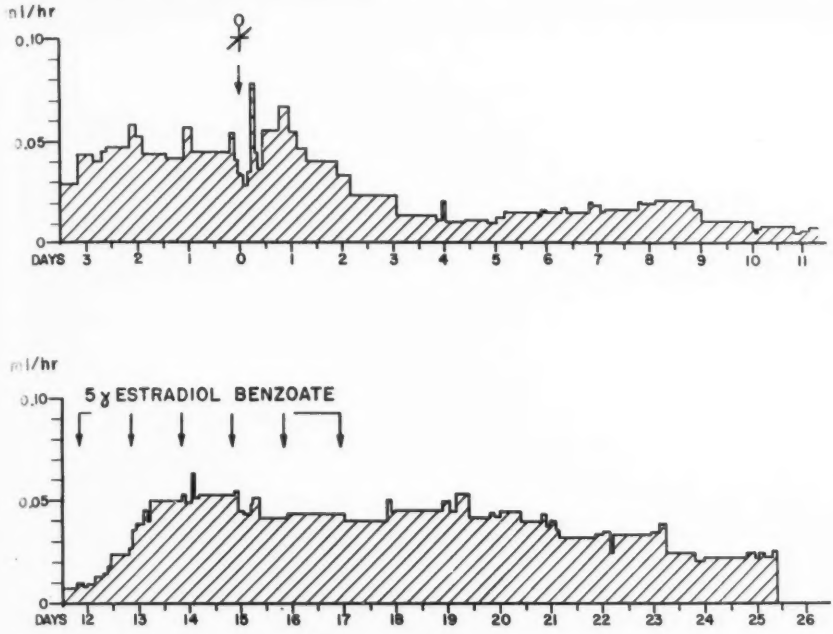

FIG. 3. Oviduct secretion rate (ml./hr.) following castration and daily postcastration administration of 5 μg. of estradiol benzoate s.c. for six days.

nificant by analysis of variance ($P = 0.05$). At necropsy, the ovaries were inspected, and corpora lutea were not found.

The Progesterone Treated Castrate. Eleven castrates were observed until the secretion rate was maintained at a postcastration plateau for several days. Progesterone, 1 mg. in 1 ml. of sesame oil, was then administered twice daily for three consecutive days. In 7 animals, the collecting system remained functional during treatment and for at least 4 days subsequent to treatment. The secretion rate remained essentially unchanged (Table 3).

TABLE 2. OVIDUCT SECRETION RATES (ML./24 HR.) AMONG NINE INTACT RABBITS TREATED WITH A SINGLE SUBCUTANEOUS INJECTION OF 3 MG. OF CRYSTALLINE PROGESTERONE/0.1 ML. OF SESAME OIL IN THE EIGHTH 24 HOUR PERIOD

24 hr. periods	1	2	3	4	5	6	7	8*	9	10	11	12	13	14
Animal No.														
1	1.38	1.41	1.50	1.51	1.45	1.36	1.31	1.34	1.29	0.98	1.36	1.52	1.72	1.62
2	2.00	2.88	2.80	2.72	2.59	2.36	2.45	2.48	1.76	1.43	1.43	2.04	2.36	2.35
3	1.30	1.29	1.24	1.24	1.36	1.19	1.24	1.42	1.12	1.14	1.58	1.69	1.68	1.67
4	1.27	1.20	1.15	1.20	1.30	1.26	1.17	1.14	1.11	0.98	1.12	1.22	1.11	1.15
5	1.58	1.39	1.28	1.46	1.26	1.24	1.28	1.52	1.50	1.54	1.64	1.76	2.00	1.76
6	1.13	1.11	1.08	1.10	1.03	1.23	0.88	1.12	1.12	0.99	0.91	0.94	1.08	1.16
7	0.92	0.96	1.07	1.28	1.26	1.12	1.11	0.96	0.80	0.69	0.76	0.66	0.61	0.66
8	0.77	0.79	0.80	0.92	0.92	0.82	0.72	0.47	0.34	0.34	0.61	0.79	0.97	0.97
9	1.01	1.08	1.08	1.16	0.95	1.03	0.99	0.94	0.89	1.27	1.28	1.11	1.39	1.23
Mean	1.27	1.37	1.34	1.40	1.35	1.29	1.28	1.26	1.12	1.04	1.19	1.31	1.44	1.40

* 3 mg. progesterone.
For $P = 0.05$, the just significant difference between two means is 0.21.

TABLE 3. DAILY SECRETION VOLUMES (ML./24 HR.) OF SEVEN CASTRATES, BEFORE, DURING, AND AFTER TREATMENT WITH 1 MG. OF CRYSTALLINE PROGESTERONE/1 ML. OF SESAME OIL TWICE DAILY

	Progesterone in castrates									
24 hr.	1	3	4	Progesterone Rx			7	8	9	10
				4	5	6				
Animal No.										
1	.42	.36	.35	.31	.26	.24	.23	.22	.27	.42
2	.39	.40	.40	.46	.33	.35	.31	.27	.32	.26
3	.19	.23	.22	.14	.10	.16	.09	.01	.15	.12
4	.65	.71	.65	.67	.67	.82	1.13	1.08	.97	1.10
5	.36	.42	.51	.46	.48	.47	.09	1.79	1.16	.91
6	.56	.51	.51	.41	.35	.41	.36	.27	.25	.31
7	.72	.80	.50	.83	.67	.46	.42	.25	.21	.21
Mean	.47	.48	.45	.47	.41	.41	.38	.55	.48	.49

For P = 0.05, the just significant difference between two means is 0.27.

Analysis of variance failed to disclose a statistical difference in the 24-hour secretion rates during the period of observation.

The Progesterone Treated Estrogen Primed Castrate. Six castrates were primed with daily subcutaneous injections of 4 μg. of estradiol 17β. After the secretion rate had displayed the maximun response to estrogen, 3 mg. of crystalline progesterone in 0.1 ml. of sesame oil were administered daily for 1 to 4 days. In each, the secretion rate decreased following treatment. Response to a given dosage schedule was quantitatively variable, however. One rabbit, primed with 8 μg. of estradiol 17β daily, failed to exhibit a decrease in the secretion rate after three consecutive daily injections of 3 mg. of crystalline progesterone in 0.1 ml. of sesame oil. It was thought that the variability in response to progesterone might be related in part to lack of uniformity of absorption. The amount of vehicle was therefore increased.

TABLE 4. DAILY SECRETION VOLUMES (ML./24 HR.) AMONG FIVE CASTRATES PRIMED WITH 2 μG. OF ESTRADIOL 17β DAILY AND TREATED WITH 1 MG. OF CRYSTALLINE PROGESTERONE/1 ML. OF SESAME OIL TWICE DAILY FOR THREE DAYS

	Est.	Est.	Est.	Est. and Prog.	Est. and Prog.	Est. and Prog.	Est.	Est.	Est.	Est.
24 hr. period	1	2	3	4	5	6	7	8	9	10
Animal No.										
1	2.19	1.90	2.51	2.20	1.77	1.47	1.56	1.92	2.05	2.3
2	0.96	1.01	1.11	1.00	0.81	0.73	0.79	0.86	0.98	1.1
3	1.26	1.75	1.24	1.40	1.06	0.96	0.91	1.02	1.12	1.1
4	1.72	1.77	1.70	1.76	1.26	1.24	1.24	1.48	1.26	1.6
5	0.88	1.27	1.53	1.46	1.21	0.95	1.07	1.36	1.46	1.6
Mean	1.40	1.54	1.62	1.56	1.22	1.07	1.11	1.33	1.37	1.5

For P = 0.05, the just significance difference between two means is 0.14.

Five castrates were primed with 2 μg. of estradiol 17β daily beginning on the second or third postoperative days. On the sixth day of estrogen treatment 1 mg. of crystalline progesterone in 1 ml. of sesame oil was administered twice daily for three consecutive days. The secretion rate decreased reaching its lowest level on the third day of treatment (Table 4). The decrease was evident on the first day of treatment. The continued fall on the second and third days was statistically significant by analysis of variance ($P = 0.05$).

DISCUSSION

A system for prolonged volumetric collection of oviduct secretions has provided evidence that the secretion rate of the rabbit oviduct is under hormonal control. Following ovariectomy the secretion rate fell to about one-third of the precastration rate. Thus, two-thirds of the secretion volume appeared to be dependent on the ovary. A decreased secretion rate following castration would be expected from the morphological changes in the secretory cells described by Borell *et al.* (4). In their study, secretory granules were scarce in the oviduct of the castrate. The possibility remains that a portion of the fluid obtained from the oviduct after castration was a transudate.

After castration the secretion rate continued to decrease over a period which averaged 67 hours. This time lag was consistent with the gradual disappearance of endogenous estrogen. Thereafter, the secretion rate was steady for several days, being maintained at a lower plateau. Later the rate began to diminish very slowly, as a result of gradual atrophy of the oviduct.

Following castration, the secretion rate could be brought to precastration levels by administration of estrogen. The prompt response to estrogen would indicate that the decreased production of secretions after castration did not result from surgical trauma to the oviduct. As little as 0.5 μg. of estradiol 17β was sufficient to cause an increased fluid production. The sensitivity of the reproductive tract to smaller doses of estrogen than are usually employed has been pointed out by Noyes *et al.* (10). They produced a physiological equilibrium resembling estrus by daily administration of 1 μg. of estradiol benzoate.

Progesterone administered in the presence of estrogen was associated with a decrease in the secretion rate. This effect was demonstrated in the intact animal. Because the status of the ovaries at the time of treatment could not be assessed, the progesterone experiments were extended to the castrate. Progesterone failed to affect the oviduct secretion rate of the castrate. In the estrogen primed castrate, consistent results were obtained when the amount of the vehicle (sesame oil) was increased to 1 ml./mg. of progesterone. The secretion rate decreased during and after treatment with progesterone (1 mg. of crystalline progesterone in 1 ml. of sesame oil twice

daily for three days) in animals primed with 2 μg. of estradiol 17β daily. The importance of vehicular volume has been stressed by Wu and Allen (11). These workers succeeded in maintaining pregnancy in ovariectomized rabbits with 1 mg. of progesterone in 1 cc. of sesame oil twice daily. Previous efforts to maintain pregnancy using a similar progesterone dose but smaller amounts of vehicle were not uniformly successful.

The effect of progesterone on oviduct secretion rate would account for the observation of D. W. Bishop, who reported decreased fluid accumulation in oviducts of pregnant rabbits after ligation at both ends (5). The unlikely possibility that the progesterone effect was mediated through the anterior pituitary gland has not been excluded.

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PITUITARY OCULAR GRAFT FUNCTION IN THE RAT, WITH A COMPARISON OF THE QUALITATIVE NATURE OF TSH FROM NORMAL PITUITARIES AND OCULAR GRAFTS[1]

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ABSTRACT

After transplantation to the anterior eye chamber of hypophysectomized or intact male hosts, neonatal rat pituitary glands exhibited evidence of TSH secretion only. Pars intermedia had no effect on adenohypophyseal function and was without detectable effect when grafted alone. TSH concentration of ocular grafts was similar to that of normal glands; unlike normal glands, however, it was not depleted by PTU feeding and did not rebound to supranormal levels after PTU withdrawal.

Hypophysectomized test animals were injected with TSH from ocular grafts and that from normal glands over a range of 2–300 mu./day. TSH from both sources produced qualitatively and quantitatively similar effects on thyroid weight and T/S ratio. The results are discussed with respect to neural control of TSH and the hypothesis of separate growth and metabolic TSH principles.

INTERFERENCE with the normal anatomical relationships between the pituitary gland and the central nervous system results in a disability on the part of the adenohypophysis in supplying increased amounts of thyrotropin (TSH) under a variety of conditions which normally evoke pituitary thyroid responses (goitrogen feeding, hemithyroidectomy, cold exposure). In animals with certain hypothalamic lesions (1–3) or with pituitary glands transplanted to the anterior chamber of the eye (4, 5) thyroid weight may be reduced to levels found in hypophysectomized animals while radio-iodine uptake and T/S ratio are maintained at high levels; such animals respond to goitrogens or partial thyroidectomy (6) with only increased radio-iodine metabolism. From this apparent dichotomy in thyroid response has developed an hypothesis of two separate TSH factors with the growth factor requiring intact hypothalamo-pituitary connections (2). A major emphasis of the work directed against this hypothesis has been to demonstrate that the T/S ratio (7–11) and radio-iodine uptake (12) respond more sensitively to thyrotropin than does thyroid weight. Accepting this evidence, it is concluded that in lesioned rats or those with ocular grafts, basal secretion of TSH or the augmentation of TSH secretion in response to propylthiouracil (PTU) is subnormal

Received June 15, 1960.

[1] Supported by USPHS grant A-3648 and NSF grant G-9814.

and of such magnitude that only radio-iodine metabolism is influenced. Recent work of D'Angelo (13), demonstrating that the pituitary TSH concentration and serum TSH levels are subnormal in lesioned animals, supports this interpretation. Evidence of increased sensitivity of radio-iodine versus thyroid weight would appear to satisfy the argument against two TSH factors according to the original thesis of Greer, namely, that secretion of growth TSH is entirely dependent upon hypothalamic regulation while metabolic TSH is regulated only at the pituitary level. However, it would only be necessary to hypothesize that two TSH factors do exist and that both are dependent upon hypothalamic influence with the growth factor being relatively more dependent; destructive hypothalamic lesions or transplantation of the pituitary would then interfere completely with growth TSH and permit only limited secretion of a metabolic component. The absence of thyroid weight change in response to PTU could then not be explained only by relative sensitivity of these two parameters of gland function. Experiments to demonstrate the greater sensitivity of the T/S ratio or I-131 uptake to endogenous TSH by using PTU blockade and graded doses of thyroxine (12) do not conclusively argue against two TSH factors since it could be further postulated that the secretion of growth factor is more easily inhibited by thyroxine.

The present experiments are designed to investigate the qualitative nature of the TSH synthesized in pituitary glands located in their normal anatomical site and in those removed from hypothalamic influence by transplantation to the anterior ocular chamber. If a central nervous system influence is necessary for formation and secretion of the growth component of a TSH complex, then TSH from glands removed from central nervous system influence should exhibit qualitative and quantitative effects different from that synthesized in normally situated glands. If no difference exists, TSH of ocular graft pituitaries and that of normal glands should produce similar metabolic and growth effects when tested at equivalent doses.

MATERIALS AND METHODS

Pituitary tissue used for ocular transplantation was of two sources: a) autografts in in which the hypophysis was removed by standard hypophysectomy technique except that ice-cold saline was used to aspirate the gland from the sella turcica; one lobe of the adenohypophysis was then weighed and transplanted to the anterior eye chamber; b) one to two-day old neonatal Sprague-Dawley rats were decapitated, their pituitary glands removed and transplanted to the anterior chamber of either intact or hypophysectomized Sprague-Dawley hosts. Sacrificing neonatal rats with either chloroform or ether was found to have a deleterious effect upon the viability of the pituitary and its ability to "take" after transplantation. An initial study was made of the histological response of the graft, animals being sacrificed, 2, 5, 13 and 30–45 days after implantation. In the remainder of the experiments, grafts were allowed to grow for 30 to 100 days with the animals maintained on Purina chow. Hypophysectomized animals were supported with 2.5% glucose drinking water for the first five days. PTU was administered in

powdered Purina chow at a concentration of 0.2%. Thyroidal I-131 uptake was measured 24 hours after intraperitoneal administration of 10 μc of carrier free I-131, the neck and hind limb regions being counted with a flat scintillation crystal. The neck-minus leg counts expressed as per cent of the administered dose represents thyroid uptake. In those experiments where I-131 uptake and T/S ratio were both measured, uptake was determined 12–18 days before autopsy. At autopsy, thyroid glands were weighed, one lobe being used for determination of the T/S ratio (14), the other lobe either fixed in Bouin's solution for histological study or used for determination of I-127 content (15). The latter method was used also for PBI measurements.

Ocular grafts used for histological purposes were prepared by fixation of the ocular lobe in Bouin's fluid after removal of its posterior half leaving the lens in place. Twenty-four hours after fixation the lens was generally removed easily without disturbance to the iris or pituitary graft. Serial sections were cut in a frontal plane at 5–7 μ and stained with aldehyde fuchsin and the modified (long) Masson trichrome procedure. Ocular grafts used for TSH analyses were obtained by first cutting along the corneal-scleral junction and removing cornea, iris and adherent graft. Under suitably high magnification the easily recognizable pituitary tissue was separated from the iris, weighed, pooled, homogenized in 0.25% acid saline and frozen. Pituitary glands from the sellae were treated in a similar fashion except that pars intermedia and pars nervosa were separated by blunt dissection and discarded. Estimation of TSH activity of the ocular graft, and pituitary glands was done by testing aliquots according to the chick P-32 uptake method of Greenspan, et al. (16) and the I-131 release method of Gilliland and Strudwick (17). Quantitative and qualitative comparison of TSH from ocular grafts and intact pituitary glands was performed by subcutaneous injection of graded doses of TSH preparations over a range of 2 to 300 milliunits per day for five days to 100 gram, hypophysectomized male rats beginning one day after hypophysectomy. Five recipient animals were used per group in all cases except the group receiving 300 milliunits of ocular graft TSH in which group 4 animals were used. At sacrifice, thyroid weights and T/S ratios were determined and one lobe fixed in Bouin's solution for histological study. Ocular grafts and pituitaries of 435 adult male hosts were used for this aspect of the study.

Corticotropic activity of pituitary ocular grafts was assessed by measurement of adrenal weight and plasma free corticoids in hypophysectomized hosts; rats were decapitated and 2 ml. aliquots of plasma used for corticoid determinations according to Guillemin *et al.* (18).

RESULTS

Growth of the ocular graft. Initial experiments indicated that transplantation of neonatal pituitary glands rather than autografts or adult homografts resulted generally in a more consistent number of successful pituitary "takes" exhibiting functional activity. For this reason, growth and histological appearance of the neonatal pituitary graft was studied in more detail. Within 12 hours after bloodless transplantation the gross appearance of the pituitary gland was altered markedly, its normal pale pink color being replaced by a deep red or black necrotic patch on the iris. The histological appearance of the graft at two days was marked by considerable cellular necrosis of the adenohypophyseal implant and a very conspicuous proliferative response of the capillary and connective tissue elements of the iris. Among the clusters of surviving adenohypophyseal cells acidophiles were recognized but basophiles or any aldehyde fuchsin positive cells could not be identified. The uveal connective tissue response reached its maxi-

mum at 5 to 6 days (Fig. 1); parenchymal cells of the graft present at this time were uniformly small, spherical with quite distinct cell boundaries and were organized in clusters of 5–6 cells and short cords separated by relatively wide zones of connective tissue containing an abundant capillary plexus. No chromophiles were present. Although planimetric studies were not performed, it is estimated that less than 20% of the original graft tissue survived. After 13 days of growth there had occurred striking hyperplasia of the surviving pituitary tissue. Thickened anastomosing cords of cells were present with a corresponding decrease in relative amount of con-

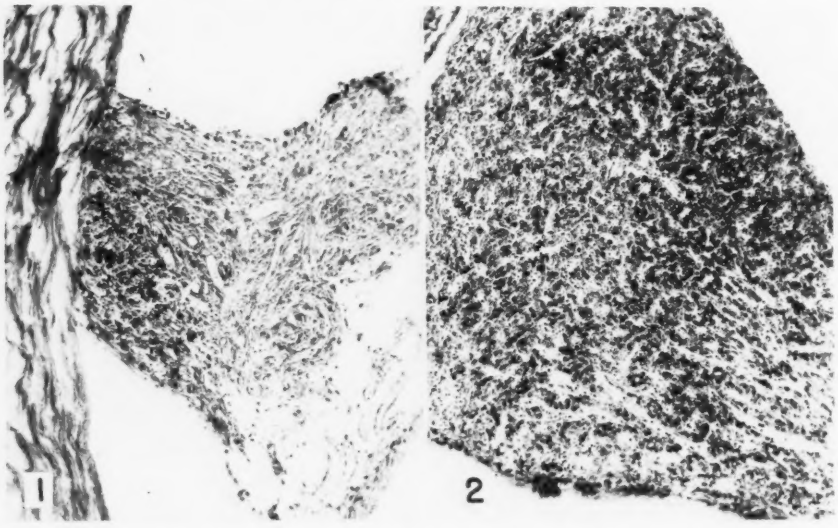

FIG. 1. Neonatal pituitary ocular graft 5 days after transplantation. Cornea at left. The cluster of adenohypophyseal tissue contains numerous pycnotic cells and represents approximately one-fifth of the original implant. The normally delicate iris (lower right corner) has proliferated markedly, invading the graft with a rich capillary plexus. Masson and aldehyde fuchsin. ×125.

FIG. 2. Neonatal pituitary ocular graft 40 days of age. Growth of the surviving pituitary tissue results in a graft approximately twice the size of the original implant, with cords of cells and sinusoids organized as in normal glands. ×125.

nective tissue. By 30–45 days parenchymal cells were present in organized cords with surrounding sinusoids similar to the organization in normal adenohypophyseal tissue (Fig. 2). Limited amounts of connective tissue existed throughout the graft, while greater amounts formed a capsule at the periphery of the graft and at sites of attachment to iris or cornea. When stained with aldehyde fuchsin—Masson, the majority of graft cells were revealed as small to medium sized chromophobes. Significant amounts of acidophilic granulation were not seen in any cells, regardless of age of the graft. Basophiles ranging in size from 9–15 μ in diameter were recog-

ized because of their notable amounts of green floccular cytoplasm; no ldehyde fuchsin positive basophiles were observed (Fig. 3). In three ifferent grafts a differential count (500 cells per graft) indicated a relative umber of basophiles of approximately 0.4%.

Cells of the pars intermedia exhibited extensive mitotic activity and yperplasia when transplanted to the anterior eye chamber. Except for bundant numbers of mitotic figures, cells of grafted pars intermedia were ot notably different histologically from those of normal glands. Pars ervosa was generally the site of extensive phagocytic activity, the neural lements being replaced completely by dense connective tissue. Occasionally a distinctive mass of epithelioid cells was present. Growth of the cular graft was additionally documented by estimating weight of the ituitary at the time of transplantation and weight of the graft 60 days later. The mean weight of 188 pituitary glands of 1–2 day old donor animals was 0.57 ± 0.02 mg. Two glands (mean weight 1.14 mg.) were implanted in one eye of each host animal. The mean weight of 84 ocular grafts after 60 days of growth was 2.78 ± 0.24 mg. Autografts did not exhibit a similar growth response. In Exp. IV (Table 1), the mean weight of 45 pituitary lobes was 3.80 ± 0.42 mg.; after 56 days of growth, mean ocular graft weight was 2.41 ± 0.38 mg.

Function of the ocular graft. Table 1 summarizes four experiments in which relative effects of autografts and neonatal pituitary glands on several parameters of thyroid function were examined. Using neonatal donor tissue, the incidence of successful "takes" was greater than 90% with each graft exhibiting evidence of functional activity with respect to TSH secretion. Autografts, in contrast, exhibited a lesser degree of successful "takes" (70–75%); furthermore, in 7 of 18 (Exp. II) and 31 of 45 (Exp. IV) grafted animals, evidence of functional activity (TSH secretion) was absent in spite of histologically identified graft tissue similar in all respects to active tissue. No explanation is available for this high percentage of autografts which were functionally inactive. The functional capacity of varying amounts of graft tissue as well as the role of pars intermedia in adenohypophyseal function was studied. After 96 days of growth, ocular graft tissue derived from five original neonatal implants per eye in 14 animals was not more active than two original neonatal glands (10 animals), T/S ratios being 34 ± 5 and 38 ± 4 respectively. Weight of the ocular graft derived from the five neonatal implants was 2.65 ± 0.38 mg., compared with 2.78 ± 0.24 mg. for grafts derived from 2 glands. After separation of the pars distalis from the pars intermedia-pars nervosa component of neonatal glands, these pieces were implanted separately. Ocular grafts composed of pars distalis only were as effective as whole glands as judged by their effect on T/S ratio, I-131 uptake and PBI. Pars intermedia-pars nervosa developed into an abundant graft which exhibited no detectable effect in hypophysectomized host animals.

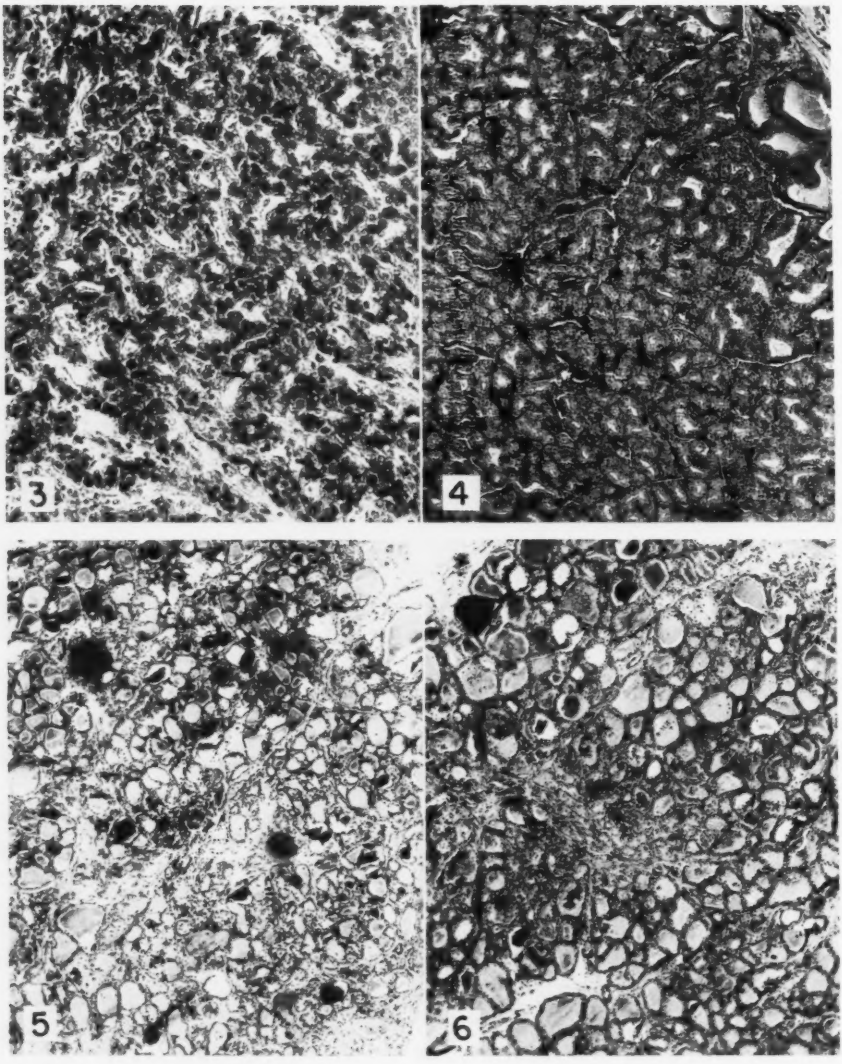

FIG. 3. Higher magnification of the ocular graft of Fig. 2.; medium-sized, lightly stained basophiles are indicated with pointers. Acidophiles or aldehyde fuchsin positive basophiles are absent. ×375.

FIG. 4–6. Thyroid glands of normal, hypophysectomized and hypophysectomized animals bearing neonatal pituitary ocular grafts respectively. Functional activity in the thyroids of grafted animals is indicated by partial restoration of follicular cell height and absorption vacuoles in the colloid. Masson stain. ×125.

TABLE 1. INFLUENCE OF PITUITARY OCULAR GRAFTS ON SEVERAL PARAMETERS OF THYROID FUNCTION IN HYPOPHYSECTOMIZED MALE HOSTS

Group	No.	Body weight, g.		Thyroid gland				PBI μg./100 ml.	Adrenal, mg.	Testes, mg.
		I.	F.	Wt., mg.	I-127 μg./100 mg.	I-131 % A.D.	T/S			
Exp. I. Autografts—35 days										
Control	9	190 ±7	325 ±11	13.0 ±2.1	75.1 ±5.2	9.3 ±1.8	46 ±4	4.3 ±0.2	43.0 ±3.0	2744 ±88
Hyp.	10	188 ±4	180 ± 6	8.1 ±1.0	71.3 ±3.7	2.8 ±0.6	15 ±1	2.4 ±0.1	11.9 ±0.7	361 ±38
Graft	12	187 ±4	222 ± 7	9.8 ±1.1	97.5 ±5.8	5.3 ±1.0	41 ±3	3.7 ±0.2	23.1 ±1.6	358 ±47
Exp. II. Autografts—48 days										
Control	8	180 ±5	252 ± 9	14.0 ±1.4	77.7 ±3.5	8.3 ±2.2		3.2 ±0.3	62.5 ±4.3	
Hyp.	8	186 ±4	178 ± 7	8.5 ±0.7	71.3 ±4.2	1.6 ±0.3		1.5 ±0.2	16.3 ±1.1	
Graft (A)	11	183 ±5	180 ± 5	8.6 ±0.8	81.2 ±3.1	3.2 ±0.9		2.1 ±0.2	17.4 ±2.0	
Graft (B)	7	190 ±6	171 ± 4	7.9 ±1.0	101 ±5.0	1.6 ±0.6		1.7 ±0.2	16.0 ±1.8	
Exp. III. Neo-natal grafts—60 days										
Control	8	187 ±9	292 ±12	13.3 ±1.4		10.0 ±1.4			50.5 ±3.8	
Hyp.	9	191 ±8	218 ± 5	8.4 ±0.9	88.2 ±4.7	0.8 ±0.1			17.4 ±1.4	
Graft	16	184 ±6	210 ± 5	7.7 ±0.9	117 ±6.1	2.1 ±0.3			18.1 ±2.1	
Exp. IV—56 days										
Control	8	194 ±8	335 ±11	12.5 ±2.0	65.0 ±4.1		54 ±4	3.0 ±0.5	56.2 ±4.7	2803 ±94
Hyp.	10	181 ±7	176 ± 4	7.2 ±0.5	95.5 ±4.1		4 ±0.4	1.4 ±0.3	11.7 ±1.3	421 ±30
Autograft (A)	14	187 ±8	189 ± 5	9.8 ±0.8	97.8 ±5.0		32 ±3	2.2 ±0.5	19.5 ±2.3	374 ±34
Autograft (B)	31	174 ±8	154 ± 6	9.1 ±1.0	63.7 ±3.7		7 ±0.5	1.3 ±0.2	18.3 ±1.8	404 ±40
Neo-natal	18	177 ±6	160 ± 4	8.5 ±0.7	91.0 ±4.2		28 ±4	2.0 ±0.2	18.5 ±2.0	429 ±52

Hyp. = Hypophysectomized. Values represent Mean ± Stand Deviation.

Functional activity of both autografts and neonatal grafts was restricted to the secretion of TSH. Hypophysectomized host animals did not exhibit consistent and significant increase in body weight; except for Exp. I (Table 1), no gonadotropic or corticotropic activity was present as judged by testicular or adrenal weight. Peripheral free corticosteroids in 16 grafted animals of Exp. III (Table 1) was 5.8 ±0.6 μg./100 ml. as compared to 5.4 ±0.4 μg./100 ml. in 9 hypophysectomized controls. Thyroid weight of the host animals was not consistently maintained at a significant increase above the hypophysectomy level. Histologically, however, follicular cell height and activity were notably influenced (Fig. 4–6). Follicular cell height in normal, hypophysectomized, and hypophysectomized-grafted animals was 12.6 ±0.6, 3.0 ±0.1 and 8.3 ±0.6 μ respectively. A consistent tendency toward a greater thyroidal I-127 concentration was noted in the

TABLE 2. INFLUENCE OF PTU ON THYROID RESPONSES IN INTACT AND HYPOPHYSECTOMIZED HOSTS WITH NEONATAL PITUITARY GRAFTS

Group	No.	Body weight, g.		Thyroid gland		
		I.	F.	Wt., mg.	I-127, μg./100 mg.	T/S
Intact-Graft	5	206 ±4	331 ± 8	15.1 ±1.9	51.5 ±1.9	45 ± 7
Intact-Graft-PTU	8	211 ±4	327 ±10	63.4 ±5.2	0.58 ±0.12	246 ±12
Hyp.	9	216 ±5	209 ± 6	10.0 ±0.9	52.6 ±8.0	5 ± 0.4
Hyp.-PTU	11	197 ±5	210 ± 8	11.6 ±0.7	60.5 ±5.0	6 ± 0.3
Hyp.-Graft	15	208 ±4	204 ± 5	10.1 ±1.0	35.9 ±2.5	39 ± 3
Hyp.-Graft-PTU	18	201 ±6	216 ± 8	12.7 ±0.8	39.7 ±4.1	135 ±10

Hyp. = Hypophysectomized. Values represent Mean ± Standard Deviation.

majority of experiments. Twenty-four hour I-131 uptake was returned to 21, 38 and 56% of normal in three separate experiments; T/S ratio was increased to an average of 78% of normal (range, 52–94%) in six different experiments. Functional activity of the thyroid was reflected furthermore in a significant elevation of the PBI level to approximatly 75% of normal which was surprisingly high in view of the fact that the ocular graft did not apparently secrete enough TSH to effect any weight maintenance of the thyroid.

In intact animals a regimen of dietary PTU evoked a pituitary TSH secretion which quadrupled thyroid weight, depleted thyroidal I-127 and produced a six-fold increase in T/S ratio (Table 2). These changes were accompanied by marked increase in thyroid follicular cell height, decrease in colloid and distortion of normal follicle shape. These parameters of thyroid function were completely unresponsive to PTU in hypophysectomized animals. Hypophysectomized rats bearing neonatal pituitary ocular grafts responded to PTU without significant influence upon thyroid weight, histology or I-127 content; T/S ratio, however, exhibited a three to four-fold

increase (Table 2). Alterations in the rate of synthesis of pituitary TSH as evoked by PTU was further reflected by the content of thyrotropin stored in the pituitary gland. Normal rat adenohypophyses contained 13.4 mu./mg. (Table 3); after 30 days of PTU, the concentration of TSH fell to 3.5 mu./mg. If PTU was withdrawn from the diet a rebound phenomenon occurred, involving an apparent decrease in release of TSH while synthesis continued. Ten days after PTU withdrawal, TSH concentration rose to 37.0 mu./mg. Ocular graft pituitary tissue did not exhibit these excursions in TSH concentration attendant upon changes in dietary PTU. When grown in either the intact or hypophysectomized host, pituitary ocular grafts contained 16.2 and 19.1 mu./mg. respectively (Table 3). Thirty days of continued PTU or 10 days of withdrawal from PTU produced no significant change in ocular graft TSH concentration.

TABLE 3. INFLUENCE OF PTU ON PITUITARY AND NEONATAL OCULAR GRAFT TSH IN INTACT AND HYPOPHYSECTOMIZED HOSTS

Group	No.	Treatment, days		Thyroid gland		Pituitary		Ocular graft	
		PTU	Withdrawal	Wt., mg.	T/S	Wt., mg.	TSH, mu./mg.	Wt., mg.	TSH, mu./mg.
Intact	6	—	—	15.7±2.1	51± 5	9.2±0.8	13.4±1.2		
Intact	5	30	—	67.6±5.7	217±18				
Intact	8	20	10	34.8±3.4	38± 8				
Intact-Graft	8	—	—	16.0±1.8	48± 6			2.31±0.12	16.1±2.0
Intact-Graft	10	30	—	61.7±6.2	209±12	10.8±0.7	3.5±0.2	2.28±0.11	25.0±1.7
Intact-Graft	10	20	10	22.4±1.8	44± 7	10.5±0.7	37.4±2.4	2.42±0.12	17.8±1.4
Hyp.-Graft	12	—	—	10.6±0.9	37± 4			2.51±0.08	19.1±2.0
Hyp.-Graft	10	20	10	11.2±1.1	30± 6			2.25±0.10	22.3±1.4

Hyp.—Hypophysectomized. Values represent Mean ± Standard Deviation.

Comparative effects of pituitary and ocular graft TSH. Thyrotropin synthesized and stored in normal rat pituitary glands and that from pituitary ocular grafts were compared with regard to their relative metabolic (I-131 metabolism) and growth stimulating properties. Neonatal glands were implanted in intact hosts and allowed to grow for 60 days. After determination of the TSH concentration in the normal and grafted pituitaries, equal doses ranging from 2 to 300 mu. per day were administered to 100 gm. hypophysectomized test animals for five days. A minimum dose of 2 mu. per day of each TSH preparation produced detectable increases in T/S ratio. A straight log dose response curve was produced up to 150 mu. per day after which a plateau appeared to be reached (Fig. 7). Thyroid weight of the test animals was not affected by doses of TSH up to 30 mu./day; between 60 and 300 mu./day, TSH from normal glands and that from ocular grafts produced equal and significant increases in thyroid weight. Ten days after withdrawal from PTU, TSH from pituitary glands was tested at 30 and 150 mu./day. Both T/S ratio and thyroid weight were influenced equally and in a fashion similar to that produced by TSH from pituitaries of animals not fed PTU.

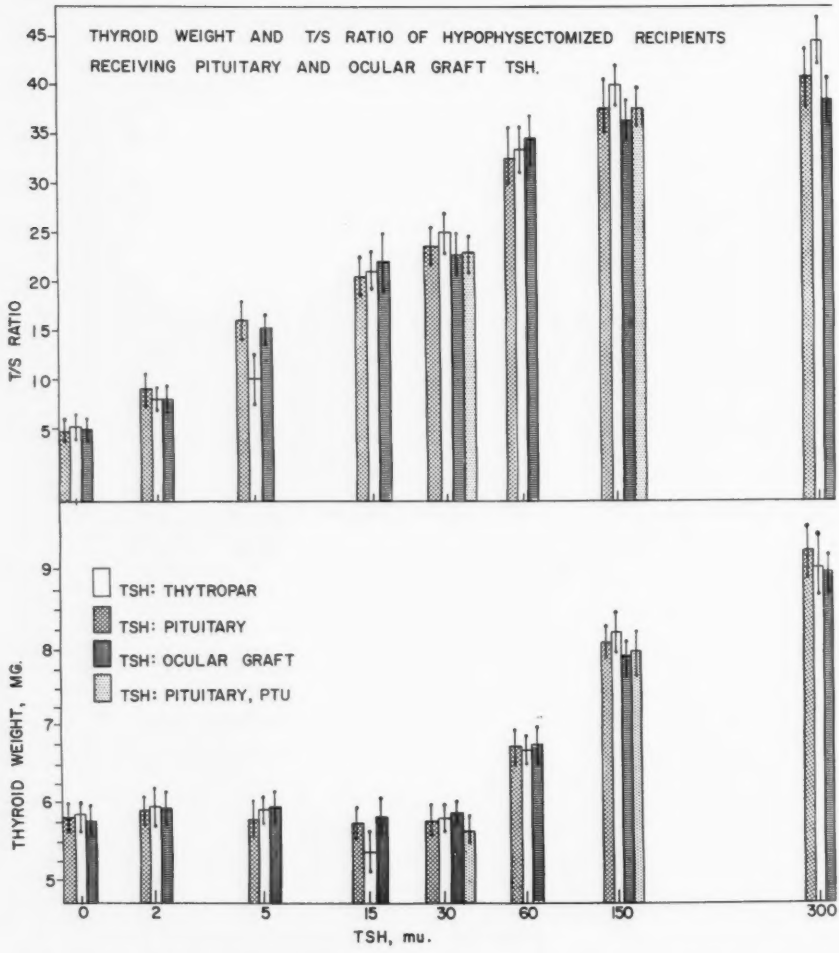

FIG. 7. The daily dose of TSH administered to 5 animals per group for 5 days is indicated on the horizontal axis. Mean T/S ratio and thyroid weight responses produced at each dose by the several TSH preparations are shown by vertical bars in the upper and lower sections of the chart respectively; standard deviations of the means are shown at the top of each bar. Pituitary TSH from animals 10 days after withdrawal from PTU was tested at doses of 30 and 150 mu.

DISCUSSION

Morphological and functional studies of ectopic pituitary tissue have been the object of numerous investigations. In spite of the fact that specific experimental conditions have differed in the various studies, it has been quite consistently observed that functional activity of the pituitary graft is

sub-minimal with respect to all of the pituitary hormones with the possible exception of luteotropin. Evidence of TSH and ACTH secretion has been noted by most investigators (19–22), gonadotropic (FSH) activity by some (23–25) and questionable evidence of growth hormone secretion by Fry and Long (23) and Goldberg and Knobil (25). In rats with pituitary glands transplanted to the kidney capsule, Hertz (26) found significant growth compared to hypophysectomized, non-grafted controls. Desclin (27), Everett (28, 29) and Nikitovitch-Winer and Everett (30, 31) found that pituitary glands transplanted to the kidney capsule secrete luteotropin at a rate possibly greater than glands in their normal site.

Cytological studies of pituitary ocular grafts are in general agreement that chromophobic elements predominate, that acidophiles are sparsely granulated if at all, and that basophiles are frequently present (25, 32–34). Specific beta basophiles (thyrotrophs) were recognized by Goldberg and Knobil (25) on the basis of aldehyde fuchsin staining, and by Martini *et al.* (34) on the basis of angular cell shape. In the present study, TSH concentration of ocular grafts was equal to that of normal glands; no significant and consistent correlation was seen in the histological appearance of the basophiles either on the basis of cell shape or staining characteristics. Calculating total TSH content, ocular grafts in this study contained 40% of that present in the normal gland; Martini *et al.* (34) found a total TSH content 50% of normal in ocular grafts which were approximately equal in volume to normal glands.

As noted by other workers (25, 34, 35), pars intermedia exerted no specific influence upon adenohypophyseal function and exhibited proliferative activity and an apparent hypertrophy when removed from neural influence (3, 36). A majority of workers have made subjective estimates of the amount of ocular graft tissue relative to the amount of tissue originally implanted or to the size of the normal gland of the particular species used. These estimates have varied markedly, with the implanted tissue exhibiting abundant to no growth at all, and the definitive graft being correspondingly much larger or smaller than normal glands. Results of the present study give some indication that factors may exist which limit the volume as well as (or in addition to) the secretory activity of adenohypophyseal tissue grown in the anterior eye chamber. The maximum amount of ocular graft tissue which grew in either intact or hypophysectomized hosts from 2–5 neonatal glands (initial weight of 1–2.5 mg.) or from one autograft (initial weight of 3.8 mg.) was approximately 2.5 mg. Failure of greater amounts of graft tissue to grow in the anterior chamber correlates with the function evidence observed in this study and by Greer (35) that TSH secretion was not improved by implanting greater amounts of tissue. In the mouse, Siperstein and Greer (33) found that adenohypophyses of neonatal pituitary ocular grafts may attain a size greater than normal *in situ* glands after 14 months of growth.

Nikitovitch-Winer and Everett (31) and Martini *et al.* (34) have summarized and added convincing evidence that the subnormal secretory activity of ectopic pituitary tissue is referable to loss of hypothalamic influence normally mediated via the portal vessels. Florsheim and Knigge (37) have additionally shown that the residual activity which is present in ocular grafts is not dependent upon hypothalamic influence reaching it via the peripheral circulation. In addition to transplantation to the anterior eye chamber, pituitary stalk transection (38, 39) and hypothalamic lesions (40–43) may also effectively interfere with the neural influence participating in pituitary-thyroid servomechanisms. In the experience of D'Angelo (13), pituitary glands "isolated" from neural influence by rather massive hypothalamic lesions possessed nevertheless a considerable degree of autonomous activity as evidenced by their ample TSH content and an ability to exhibit supranormal "rebound" after depletion by PTU. Ocular grafts, in spite of their normal TSH concentration, exhibited no PTU-induced fluctuations in hormone content. The graft-bearing animal, however, does respond to PTU with increased T/S ratio, as noted by Scow and Greer (5). Data of this study show that this response occurs without significant depletion of thyroidal iodine content and may add additional evidence that lowered thyroxine levels can evoke increased TSH secretion at the pituitary level. Von Euler and Holmgren (44, 45), Yamada and Greer (46) and Yamada (47, 48) have demonstrated convincingly that elevated thyroxine levels depress TSH secretion at the pituitary and/or hypothalmic levels.

The principal objective of this study, a qualitative comparison of TSH from normal and ectopic pituitary glands, was initiated to clarify the problem of whether separate growth and metabolic TSH principles exist. The original thesis of Greer (2), outlined above, was subsequently modified to suggest neural control of *both* TSH factors (35), and more recently revised in favor of a *single* TSH principle (12). In the present experiments, TSH from (a) normal glands, (b) ocular grafts, and (c) normal glands subjected to fluctuations in TSH concentration by PTU feeding and withdrawal were assayed by procedures based upon "metabolic" TSH activity (P^{32} uptake; I-131 release) and compared in other assay animals for their metabolic (T/S) and thyroid weight producing ability. It was to be anticipated that the several TSH preparations would exhibit equivalent T/S responses at similar doses; the crucial parameter was, therefore, thyroid weight. When assayed in hypophysectomized test animals, the several TSH preparations were qualitatively and quantitatively similar with respect to their effect on iodine metabolism and thyroid weight. TSH from pituitary tissue of all sources produced steeper dose-response curves with T/S ratio than with gland weight. At all dose levels tested, TSH from each source produced similar thyroid weight changes. The data indicate strongly that an absolute or relative dissociation of growth and metabolic TSH principles

is not demonstrated in the stored content of TSH from pituitary glands separated from neural influence or in normal glands subjected to fluctuations in TSH concentration by PTU feeding and withdrawal, responses which may also require neural participation.

Acknowledgments

Grateful acknowledgment is made to Mr. Reginald Lawrence, Misses Ruth Greene, Betty Hall and Delores Schock for their invaluable assistance in this study.

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DIRECT ACTION OF ESTROGEN ON THE OVARY OF THE IMMATURE RAT[1]

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ABSTRACT

Crystalline estradiol or stilbestrol was applied to one ovary of the immature rat leaving the other ovary untreated. The estrogen treated ovary exhibited several unilaterally differential responses: (1) a greater increase in weight, (2) formation of corpora lutea, (3) an increased responsiveness to endogenous and exogenous gonadotrophins. In control experiments crystalline testosterone or progesterone was applied to one ovary with, or without, estradiol being placed on the other ovary. These experiments demonstrate that estrogen has a local stimulatory effect within the ovary as well as a systemic effect via the pituitary.

PENCHARZ (1) and Williams (2) found that stilbestrol pellets implanted into rats at the time of hypophysectomy not only maintained ovarian weights but also made the ovaries more responsive to exogenous gonadotrophins. Payne and Hellbaum (3) demonstrated that relatively large doses of stilbestrol (1 mg. per day) caused more ovarian growth in hypophysectomized rats than did smaller doses. Subsequently stilbestrol was utilized in hypophysectomized and intact rats to augment the ovarian responses to gonadotrophins (4, 5). These findings, especially in hypophysectomized rats, indicated that estrogen had a direct action on the ovary. The present report is concerned with experiments in which estrogen was applied directly to one ovary. In these experiments the local effects of estrogen were evident in the treated ovary, whereas the systemic effects of estrogen were evident in the opposite ovary.

MATERIALS AND METHODS

Immature Sprague-Dawley rats 25 to 30 days of age were used in this study. Under ether anesthesia the abdomen was opened and an ovary brought into view. The periovarian bursa was stripped away and 0.5 to 1.0 mg. of crystalline estrogen was applied to the surface of the ovary with a small spatula. The serosanguinous fluid on the surface of the ovary clotted and held the estrogen in contact with the ovary. The ovary was then replaced into the abdominal cavity and the abdominal incision closed with cotton sutures. These experiments were controlled by using testosterone or progesterone

Received June 17, 1960.

[1] This study was supported by USPHS Grant A-1686.

crystals unilaterally instead of estrogen. In other experiments, testosterone or progesterone was applied to one ovary and estradiol to the other, thus duplicating the factor of trauma on each side. The rats were killed on the fifth postoperative day. When gonadotrophins were employed, they were injected on the 2nd, 3rd and 4th postoperative days, and then the rats were autopsied on the 5th day. At autopsy the ovaries were stripped free of adhesions, weighed individually and fixed in Bouin's solution. Histologic sections were prepared for microscopic evaluation.

EXPERIMENTAL

Unilateral Ovarian Response to Estrogen

Estradiol was applied to the left ovaries of five rats on the 2nd day after hypophysectomy. Seven days later the estrogen-treated ovaries averaged 14.2 mg. (range 10–17 mg.) whereas the right ovaries averaged 9.4 mg. (range 9–10 mg.). Histologically the ovaries were composed of many medium-sized prevesicular follicles.

Estradiol was applied to the left ovaries of two rats on the 11th day after hypophysectomy. Five days later there was no appreciable increase in ovarian weight (5.8 and 6.6 mg. vs 3.4 and 6.4 mg.) but the increased number of growing follicles was typical of the ovarian response to estrogen.

Unilateral Ovarian Responses to Endogenous Gonadotrophin

Ten immature rats each had estradiol applied to one ovary and the rats were autopsied five days later. There were corpora lutea in six of the estrogen-treated ovaries but none in any of the contralateral ovaries. The weights averaged 24 mg. (range 18 to 40 mg.) for the treated ovaries and 11 mg. (range 10 to 16 mg.) for the control ovaries. Three rats had progesterone applied to one ovary but no corpora lutea formed and the progesterone treated ovaries averaged 13.3 mg. as compared to 10 mg. for the opposite ones.

Table 1 shows the ovarian weights after application of progesterone and/or estrogen in a group of 12 rats. Progesterone had no obvious effect on ovarian weight since the treated ovaries averaged 10.6 mg. as compared to 10.2 mg. for the control ovaries. When progesterone was applied to the left ovary and estradiol to the right in four rats, there were corpora lutea in three of the estrogen-treated ovaries at autopsy. The progesterone-treated ovaries averaged 12 mg. in weight as compared to a 21 mg. average for the estrogen-treated ovaries. After estrogen was applied to the left ovaries of four rats, two of these developed corpora lutea (Fig. 1); and these treated ovaries were about twice as heavy (23 mg.) as the opposite ones (12.5 mg.).

Unilateral Ovarian Responses to Exogenous Gonadotrophin

Three rats were opened four days after hypophysectomy and estradiol crystals were placed on the left ovaries. Two days later these rats were

TABLE 1

Body weights, grams		Steroid applied to ovary	Ovaries, mgm.	
Initial	Autopsy		Left	Right
80	105	Progesterone on left	11	11
89	112		8	8
77	100		10	9
76	92		12	11
83	92	Progesterone on left	12	26 C.L.
86	98	Estradiol on right	14	22 C.L.
77	87		9	18 C.L.
83	103		12	17
78	88	Estradiol on left	24 C.L.	13
75	90		28 C.L.	13
73	86		22	12
79	89		18	12

This group of rats was 30 days of age at the time the steroids were applied to the ovaries and 35 days of age at autopsy. C.L. denotes corpora lutea in the ovaries.

started on FSH[2] injections (total dose 0.4 mg.). At autopsy the estrogen-treated ovaries weighed 25, 36 and 50 mg. as compared to 23, 19 and 34 mg. respectively for the opposite ones. An early corpus luteum was found in one of the estrogen-treated ovaries, but the general response was the typical follicular growth and a greater number of follicles in the treated ovaries (Fig. 2).

Estradiol was applied to the left ovaries of seven rats. Then a total dose

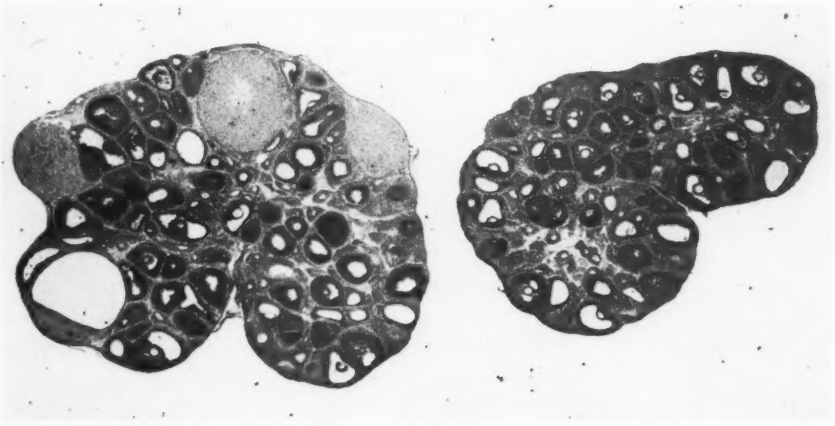

FIG. 1. Sections of ovaries from a rat with estradiol applied to the left ovary. Autopsy five days later. The estradiol-treated ovary weighed 24 mg. as compared to 13 mg. for the opposite one. In this cross-section there are three corpora lutea and one cystic follicle with marginal luteinization in the estrogen-treated ovary.

[2] The FSH was furnished by the NIH Endocrine Study Section.

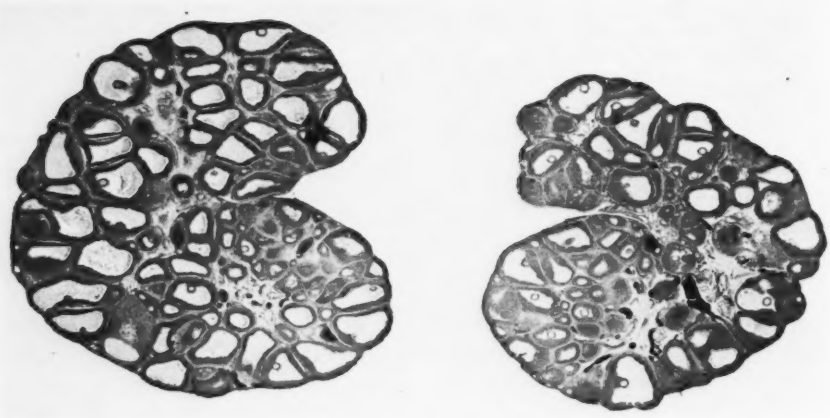

FIG. 2. Sections of ovaries from a rat that had estradiol applied to the left ovary on the fifth day after hypophysectomy. During the three days prior to autopsy the rat had received 0.4 mg. of FSH. The estradiol-treated ovary weighed 35 mg. as compared to 23 mg. for the right ovary. The difference in weight is apparently due to a larger number of vesicular follicles in the estrogen-treated ovary.

FIG. 3. Sections of ovaries from a rat with estradiol on left ovary and testosterone on right ovary. Rat had been given 20 I.U. HCG during the three days prior to autopsy. Estradiol-treated ovary weighed 57 mg. and testosterone-treated ovary weighed 17 mg. In these cross-sections there are 10 large cystic follicles in the left ovary and only one in the right. Hypertrophied interstitium is present in both ovaries.

of 0.5 mg. FSH was administered during the 2nd, 3rd and 4th postoperative days. At autopsy the estrogen-treated ovaries averaged 65.7 mg. (range 51 to 83) in contrast to 36.4 mg. (range 30 to 54) for the right ovaries. Corpora lutea were present in 5 of the estrogen-treated ovaries but none of the opposite ovaries so the FSH treatment did not alter the expected occurence of corpora lutea. After six unoperated controls were given the same amount of FSH the left ovaries averaged 47.1 mg. (range 36 to 58) as compared to 39.1 mg. (range 28 to 42) for the right ovaries. The right ovaries of the two groups thus exhibited comparable responses.

Stilbestrol was applied to the left ovaries of six intact rats and two days later injections of chorionic gonadotrophin (HCG) were started. A total dose of 20 I.U. in 72 hours resulted in weights averaging 38 mg. (range 28 to 46) for the stilbestrol-treated ovaries and 19 mg. (range 11 to 24) for the contralateral ovaries. The difference in weight was due to a larger number of cystic follicles in the estrogen-treated ovaries. In the next experiment stilbestrol (2 rats) or estradiol (3 rats) was applied to the left ovaries before giving a total dose of 20 I.U, HCG. The estrogen-treated ovaries averaged 84 mg. (range 51 to 121) in contrast to 28 mg. (range 25 to 56) for the opposite ones. Similar differences were obtained when stilbestrol was placed on one ovary and testosterone on the other and then injections of HCG were given (Fig. 3). Testosterone-treated ovaries did not differ appreciably from untreated control ovaries in these experiments.

DISCUSSION

These experiments clearly show that estrogen has a direct local effect on the ovary. In immature rats with estrogen applied to one ovary, the formation of corpora lutea in that ovary indicates it has been rendered more responsive to endogenous gonadotrophin than the other ovary. In early studies (6, 7) there was evidence that after 3 days of estrogen treatment, the pituitary of the immature rat released significant quantities of LH. In the present experiments the systemic action of the estrogen probably caused the pituitary to produce LH while the local action of estrogen on the ovary served to increase its sensitivity to LH. The absence of this LH effect in hypophysectomized rats treated with FSH was manifest in the absence of corpora lutea even though an enhanced unilateral follicular response was demonstrated.

The increased responsiveness of the treated ovary to HCG was of the same magnitude as occurred in both ovaries when stilbestrol-in-oil was administered for two days prior to starting the HCG injections (5). In each instance the response to HCG was the formation of cystic follicles, some of which showed peripheral lutenization. However the response was primarily unilateral after estrogen was applied directly to one ovary. It seems noteworthy that HCG stimulation produced such atypical luteinization that it prevented or masked the formation of normal corpora lutea

that were found in the experiments in which FSH or even estrogen alone was used.

At the time of autopsy there were vascular adhesions of periovarian tissues to the surface of the estrogen-treated ovaries. These adhesions were friable and were easily stripped away, so the observed differences in ovarian weights were not unduly altered by adherent tissue. Unabsorbed crystalline steroid was still visible on the surface of the ovary and in the adhesions, so probably local concentration was high and absorption was still active at the time of autopsy. Estrogen crystals produced more vascular adhesions than either progesterone or testosterone. The significance of the adhesions and the vascularity is unknown but the qualitative and quantitative changes in the ovary were so similar to those occurring after large systemic doses of stilbestrol (5, 8) that it seems the major effects described here are due to alterations in ovarian follicular sensitivity.

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EFFECT OF SODIUM DEFICIENCY ON SECRETION OF HORMONES BY THE RAT ADRENAL CORTEX[1]

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ABSTRACT

Previous studies have shown that when adrenals of sodium deficient rats are incubated *in vitro* there is increased production of aldosterone but reduced secretion of other Δ4,3-ketosteroid hormones including corticosterone. Other investigators have demonstrated increased aldosterone secretion after sodium restriction but reduced production of other hormones has not been described. For this reason, the effect of salt depletion on adrenocortical hormone secretion of the rat was reinvestigated by inducing sodium deficiency in animals and measuring hormone content of adrenal vein plasma. The results demonstrate that in salt-depleted rats the plasma level of corticosterone and the rate of secretion of this hormone are decreased. There is also a reduction in plasma concentration of Δ4,3-ketosteroids which is probably a result of the marked decline in corticosterone production. The fact that corticosterone secretion of sodium depleted rats is diminished whereas similar changes have not been observed in other animals indicates that the response of the rat adrenal to sodium restriction is different from that of most other species.

PREVIOUS investigation reported from this laboratory has demonstrated that sodium deficiency in rats results in reduced secretion of adrenal steroids containing a Δ4,3-ketone structure (1). Although total production of adrenocortical hormones with this configuration was diminished, chromatographic separation of individual steroids demonstrated increased secretion of aldosterone. The decline in total hormone production was accounted for by a marked decrease in synthesis and release of corticosterone, the major adrenal steroid in the rat (2). In the previous study, adrenals of sodium deficient rats were incubated with ACTH *in vitro* and steroids secreted into the medium were quantitatively determined. Other investigations of adrenal cortical function in sodium depleted animals using *in vivo* technics have demonstrated increased aldosterone production but decreased secretion of corticosterone or other steroids has not been found (3, 4, 5). Since the reduced secretion of adrenal steroids observed in our experiments occurred *in vitro* and has not been confirmed, the effect of salt depletion on adrenocortical function of rats was reinvestigated using *in vivo* technics. The results demonstrate that in salt-

Received June 20, 1960.

[1] This research was supported by a grant from the National Institute of Arthritis and Metabolic Disease, United States Public Health Service.

deprived rats production of Δ4,3-ketosteroids, including corticosterone, is reduced.

METHODS

Male Holtzman rats weighing 60–70 grams were fed a synthetic, sodium-deficient diet (6) and given distilled water to drink. A similar group of rats was pair-fed the same diet to which salt was added. On direct analysis, the deficient diet contained 19.3 meq. of sodium/kg., whereas sodium content of control food was 293 meq./kg. Average daily food consumption per rat during the experiment was 13.1 grams. Animals were housed individually in metabolism cages and once each week 18 hour urine collections for determination of sodium content were done on six rats from each group. During the final week, hematocrits as well as serum sodium and potassium concentrations were determined on tail blood from each rat. Beginning on day 51 and continuing for three additional days, cannulas were introduced into the left renal veins of three rats from each group and adrenal vein blood obtained according to the method described by Longwell (7). In each instance blood was collected as long as flow rate remained steady and the collection period was carefully timed. Plasma was separated from cells by centrifugation and stored at −20° C until hormone analyses were carried out. When collection of blood was complete, rats were sacrificed and adrenals removed, dissected free of fat and weighed.

Total content of Δ4,3-ketosteroid hormones in adrenal vein plasma was determined using the isonicotinic acid hydrazide method (8). Corticosterone concentration was measured using the fluorometric procedure of Silber (9).

RESULTS

Rats fed the sodium deficient diet gained weight more slowly than did pair-fed controls so that, at the end of the experiment, mean body weight of the depleted group was about 25 per cent less than that of the control group (Table 1). Even though body weight of deficient animals was reduced, mean adrenal weight of these rats was greater than that of controls although the difference was not marked (Table 1). When adrenal weight is expressed in relation to body weight, it is evident that hypertrophy of the gland occurred in the sodium depleted rats (Table 1). The difference in adrenal weight–body weight ratios of the two groups is significant, $P = <0.001$.

In deficient animals there was a decrease in serum sodium concentration and an increase in serum potassium and blood hematocrit when compared to controls. Mean serum sodium and hematocrit values for the two

TABLE 1

	Body weight (grams)	Left adrenal weight		Serum sodium (meq./liter)	Serum potassium (meq./liter)	Urine sodium (meq./liter)	Hematocrit (%)
		(mg.)	(mg./100 G body wt.)				
Sodium Deficient Rats (12)*	172.4±4.7	25.0±0.94	14.6±0.62	138.0±0.46	6.9±0.17	0.41±0.05	50.5±0.48
Pair-fed Controls (12)*	239.4±3.8	22.5±0.78	9.4±0.24	143.9±0.61	5.8±0.06	15.93±1.52	44.5±0.36

* Number of animals.

Body Weights, Adrenal Weights, Hematocrits and Serum and Urine Electrolytes of Deficient and Control Rats. (Mean values ± standard error of mean.)

TABLE 2

	Plasma flow (ml./hour)	Corticosterone secretion (γ/ml. plasma)	Δ4,3-ketosteroid secretion (γ/ml. plasma)	Corticosterone secretion rate (γ/hr./100 mg. left adrenal weight)	Δ4,3-ketosteroid secretion rate (γ/hr./100 mg. left adrenal weight)
Sodium Deficient Rats (9)*	5.65±1.28	8.46±1.61	14.89±1.76	139.4±17.4	315.7±79.6
Pair-Fed Rats (11)*	5.36±1.24	16.32±1.35	21.57±1.50	352.2±60.7	473.4±90.5

* Number of determinations.
Adrenal Steroid Hormone Secretion of Sodium Deficient and Pair-Fed Control Rats. (Mean values ± standard error of the mean.)

groups were significantly different, $P = <0.001$, while the difference in serum potassium levels was significant, $P = <0.01$. Throughout the experiment the urinary sodium excretion of deficient rats was very low and far less than that of rats fed the diet containing salt (Table 1).

Concentration of corticosterone in adrenal vein plasma of salt-depleted rats was approximately half that of control animals (Table 2). When corticosterone secretion is expressed as quantity of hormone produced/hour/100 mg. adrenal weight, the difference is even greater. Differences in plasma concentration and rate of corticosterone secretion of the two groups are statistically significant, $P = <0.01$. Plasma concentration of Δ4,3-ketosteroids was less in deficient than in control rats (Table 2) and the difference is statistically significant, $P = <0.01$. When production of these hormones is considered on the basis of quantity of steroids secreted/hour/100 mg. adrenal weight, there is a similar difference; however, this difference is not significant.

DISCUSSION

In a previous investigation it was demonstrated that sodium depletion in rats resulted in increased aldosterone secretion but reduced production of corticosterone and other steriods (1). Although aldosterone synthesis was enhanced by salt restriction, the decline in secretion of other steroids was so great that total production of Δ4,3-ketosteroid hormones was decreased. Other investigators have also found increased secretion of aldosterone after sodium restriction but have not observed changes in production of other adrenal steroids (3, 4, 5). Because of this, it was thought that our findings might be explained by the fact that adrenal secretion in our experiments was studied under *in vitro* conditions. To determine whether this explanation was valid, hormone analyses in the current studies were carried out on adrenal vein plasma of sodium deficient and control rats. The results again demonstrate that secretion of corticosterone is diminished and suggest that total production of Δ4,3-ketosteroids may also be reduced. The finding that the decline in corticosterone secretion is greater than that of Δ4,3-ketosteroid production is probably accounted for by the increase in aldosterone synthesis which occurs in sodium deficient animals since aldosterone is detected by the procedure used for

determination of Δ4,3-ketosteroids but not by the method for measuring corticosterone.

Since in the salt-depleted rat there is reduced secretion of corticosterone as well as increased synthesis of aldosterone, one wonders why the only change in hormone production of sodium deficient animals observed by others has been an increase in aldosterone secretion. There appear to be two possible explanations; first, it may be that sodium deprivation was unusually severe in our investigations. The fact that salt restriction was of greater duration in our experiment than in other studies (3, 4, 5) gives support to this suggestion. The decline in serum sodium as well as the extremely small amount of sodium exreted in the urine of deficient rats in this experiment attest to the severity of salt depletion. In the experiments of Singer and Stack-Dunne (3), no change in secretion of corticosterone was observed in sodium deficient rats even though aldosterone production was greatly increased. It was noted by the authors, however, that salt depletion was not marked and their data show only a slight decrease of serum sodium in their animals. Secondly, it may be that the rat adrenal response to sodium deprivation is different from that of other animals. That this may be true is suggested by the work of Rosnagle and Farrell (4), who induced sodium deficiency in dogs and found an increase in adrenal vein aldosterone but noted no change in hydrocortisone secretion. Likewise, no alteration in production of hormones other than aldosterone has been observed to result from salt restriction in man (5). Thus, the response of the rat adrenal to rigid sodium restriction appears to be different from that of man and the dog and, perhaps, from other species as well. The finding that the rat adrenal responds to salt depletion in a manner different from that of other animals is not unexpected since it is well known that normally the rat adrenal secretes mainly corticosterone whereas in most other species cortisol is the major adrenal steroid (2).

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THIOACETAMIDE, AN ANTI-THYROID COMPOUND[1]

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ABSTRACT

Thioacetamide has been found to inhibit the iodination of tyrosyl groups in the thyroid of the rat. Its rapid metabolism prevents it from being a goitrogen.

IN ASSAYING a number of compounds for anti-thyroid activity, Astwood (1) noted that thioacetamide ($CH_3 \cdot CS \cdot NH_2$) was not goitrogenic. Although it lacks the thiourylene grouping (—NH·CS·NH—), it does contain a thiocarbamide moiety, hence one might expect it to possess anti-thyroid activity. It is the purpose of this study to demonstrate that thioacetamide is an effective, but short lived anti-thyroid compound, and that its rapid metabolism precludes it from being a goitrogen.

EXPERIMENTAL

Materials: S^{35}-thioacetamide was obtained from the New England Nuclear Corporation. The specific activity was 30 millicuries/millimole. The chromatographically pure compound was dissolved in distilled water and stored at −20° under nitrogen. Radioactive iodide (I^{131}), essentially carrier free, was obtained from the Atomic Energy Commission at Oak Ridge, Tennessee.

Non-radioactive thioacetamide and thiourea were dissolved in distilled water. Thiouracil was dissolved in dilute potassium hydroxide, final pH 8.0–8.5.

Anti-Thyroid Assay: An assay for the anti-thyroid activity of various compounds has been devised which takes cognizance of two facts: 1. that the iodination of tyrosyl groups in the thyroid of the rat occurs rapidly (2), and 2. that the anti-thyroid compounds reach their site of action in the thyroid within minutes of their intraperitoneal administration to rats (3).

The compound to be tested is administered intraperitoneally to the animal. Within five minutes, 5 to 10 microcuries of carrier free radioactive iodide (I^{131}) is similarly administered. Fifteen minutes later, the animal is killed by exsanguination. The thyroid is rapidly removed, cleaned, weighed (wet weight) on a Roller Smith Torsion balance, and is homogenized in chilled distilled water. An aliquot of the homogenate is placed on Whatman #1 chromatography paper which is run in an ascending solvent system of

Received June 27, 1960.

[1] This is publication No. 73 of the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts. This work was aided by grants from the National Institutes of Health (3274 P.E.T.) and the National Science Foundation (G 5561).

n-butanol-dioxane-2N ammonia (4:1:5). Carrier iodide is also applied to the paper and is identified by spraying with palladium chloride. Two radioactive spots are resolved, one of which remains at the origin and is presumed to be protein-bound iodine (PBI^{131}) and an inorganic iodide (I^{131}) spot. The strip of paper is cut in sections which are counted for radioactivity in a well type scintillation counter. The percentage of PBI^{131} as a proportion of the total I^{131} in the thyroid is thus determined.

S^{35}-thioacetamide

Male Sprague-Dawley rats weighing 90 to 100 gm. were maintained on Purina Fox Chow diet. The S^{35}-thioacetamide was administered intraperitoneally as a single dose, 40 micromoles (μmoles). At intervals, the animals were killed by exsanguination. The thyroid and a portion of the liver were removed, cleaned, weighed (wet weight) and then homogenized in 0.5 ml. of a chilled solution of non-radioactive thioacetamide (0.002 M). Appropriate aliquots of the tissue homogenates, the serum and S^{35}-thioacetamide standards were assayed for total S^{35} radioactivity (3).

The S^{35} metabolites were characterized by paper chromatography and by paper electrophoresis (3, 4). A twenty microliter aliquot of the tissue homogenate or a five microliter aliquot of serum plus 0.3 micromole of thioacetamide carrier were applied along a 1.5 cm. line to Whatman #1 chromatography paper. The latter was run in an ascending solvent system of ethanol-1 M ammonium acetate, pH 7.5 (7.5:3). The radioactive spots along the paper strips were located by radioautography using No-Screen X-ray film. At this point, it might be well to note a technical difficulty that was encountered. It was found that the non-radioactive carrier thioacetamide reacted with the X-ray film producing a darkening which would have obscured the radioactive thioacetamide spot. This reaction probably results from the decomposition of thioacetamide to hydrogen sulfide (5). It was suggested[2] that we spray the paper chromatogram with 0.5N $AgNO_3$ prior to applying the X-ray film. This spray not only helped to identify the carrier thioacetamide as a brown-black spot, but also prevented the thioacetamide from reacting with the X-ray film.

The amount of S^{35} radioactivity in various areas was determined by cutting up the chromatogram and assaying the radioactivity as a percentage of the total number of counts along the strip.

RESULTS

Anti-thyroid Assay

Initially, various doses of thioacetamide were administered to rats to determine the dose necessary to completely inhibit the iodination of tyrosyl groups in the thyroid. It is evident from Table 1 that 40 μmoles of thioacetamide is required to inhibit this reaction. This is to be contrasted with levels of 10 μmoles for thiouracil and 20 μmoles for thiourea.

The inhibition by thioacetamide is short lived. Within two hours of the administration of 40 μmoles to animals, the amount of PBI^{131} formed by the thyroid is back to normal (Fig. 1). The effect of 20 μmoles of thiourea persists for at least four hours, and that of 20 μmoles of thiouracil for longer than five hours. It has been previously demonstrated that the effect of 40 μmoles of thiourea persists for 8–10 hours and that the effect of 40 μmoles of thiouracil persists for up to twenty-four hours (3). Note

[2] By Mr. Marshall Horwitz, a second-year Tufts Medical School student, working in the laboratory under an Institutional Cancer Grant (IN 29a) to Brandeis University.

TABLE 1. EFFECT OF THIOACETAMIDE, THIOUREA AND THIOURACIL ON FORMATION OF PBI^{131} BY THYROID OF THE RAT

Dose inhibitor, μmoles	PBI^{131} as % of total I^{131}		
	Thioacetamide	Thiourea	Thiouracil
Control	70	72	72
1.0	71	67	47
5.0	74	48	20
10.0	69	4	0
20.0	35	0	0
40.0	0	0	
60.0	0		

The data at each interval are derived from a single animal for each compound.

of these compounds alter the concentration of iodide by the thyroid in the interval of study.

The injection of 40 μmoles of thioacetamide subcutaneously every twelve hours into rats for 8 days failed to produce any increase in the weight or cell size of the thyroid as contrasted with controls. It did, however, produce a marked increase in the size of liver nuclei, a finding which has been previously reported (6).

S^{35}-thioacetamide

It is evident from Figure 2 that thioacetamide reaches the thyroid within minutes of its administration to rats, the peak concentration occurring at forty-five minutes.

Analysis of the S^{35} metabolites by paper chromatography reveals that thioacetamide is rapidly metabolized by the thyroid. Within five minutes, only 46% of the total radioactivity in the thyroid is present as thioacetamide (Table 2). At the end of two hours, the thioacetamide is completely

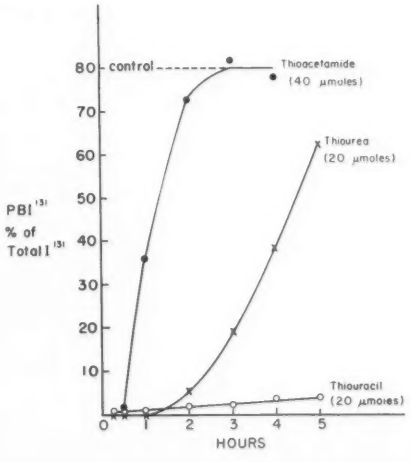

FIG. 1. The effect of a single dose of thioacetamide, thiourea and thiouracil on the formation of PBI^{131} by the thyroid of the rat over a five-hour interval. The data at each interval are derived from a single animal for each compound.

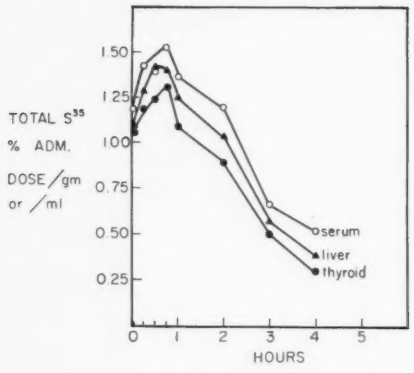

FIG. 2. The per cent of the administered dose of S^{35} radioactivity per gram of thyroid or liver or per ml. of serum following the administration of a single dose of 40 μmoles of S^{35} thioacetamide to normal rats. Data at each time interval are derived from a single animal.

metabolized. The sulfur products are protein-bound sulfur (PBS^{35}), sulfate, thiosulfate, and an unknown compound.

The disappearance of thioacetamide from the liver and serum is nearly as rapid as from the thyroid. The metabolic products are also similar to those in the thyroid except that there is 50 to 75% less protein-bound sulfur formed.

DISCUSSION

An assay of anti-thyroid activity is described which should be useful in evaluating the effectiveness of various compounds in inhibiting the iodination of tyrosyl groups in thyroid tissue. With this technique, it has been found that thioacetamide is an effective but short-lived anti-thryroid compound. Its duration of action in the thyroid lasts only about two hours following the administration of forty micromoles to rats. This factor may be of value in thyroid studies involving a series of experiments in the same animal or subject.

Thioacetamide is rapidly concentrated by the thyroid; its peak concentration occurs at forty-five minutes and is about 4.0×10^{-4} M. There is no concentration gradient for thioacetamide between thyroid and serum. This differs from the data of Nygaard *et al.* (7) which suggested that such a

TABLE 2. CHROMATOGRAPHIC ANALYSIS OF THE RADIOACTIVITY IN THE THYROID OF RATS GIVEN 40 μMOLES OF S^{35}-THIOACETAMIDE

Interval after administration	S^{35} spots, % of total S^{35} along the chromatogram			
	Rf 0.0	0.22	0.74	0.81
	PBS^{35}	"S"*	x	Thioacetamide
5 min.	5	9	36	46
30 min.	6	11	57	22
1 hour	8	40	26	22
2 hours	11	70	14	0

* A mixture of thiosulfate and sulfate as determined by paper electrophoresis (4).

gradient did exist. The concentration in thyroid tissue necessary to inhibit the iodination of tyrosyl groups is about 8.0×10^{-5} M. In these respects, thioacetamide closely resembles thiourea and thiouracil (3).

Thioacetamide is rapidly metabolized by the thyroid, about 1 μmole/gm./hr., a factor which probably explains its failure to be a goitrogen. This is in contrast to thiourea and thiouracil which are metabolized at a slower rate by the thyroid (3) and are goitrogenic. The major sulfur metabolite is sulfate.

Thioacetamide has been reported to increase the synthesis of nuclear ribonucleic acid (RNA) in the liver (8). This finding is consistent with the increase in the size of the nucleus and in its RNA content (9, 10). Similar histologic changes were not found in the thyroid.

Acknowledgments

To Miss Ellie Bougas, Mr. Marshall Horwitz and Mr. Henry Guerin for invaluable technical assistance.

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POSTPONED HOMOTRANSPLANTS OF THE HYPOPHYSIS INTO THE REGION OF THE MEDIAN EMINENCE IN HYPOPHYSECTOMIZED MALE RATS[1]

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ABSTRACT

In 2 series of Long-Evans male rats pituitary homotransplants were made by the parapharyngeal approach after pituitaryless periods of 60 and 150 days. During these periods no gain, usually a loss, in body weights and testicular atrophy occurred. Autopsies were done 2½ months after the transplantations. In all rats the transplants became functional. In Series I, hypophysectomized when 38–42 days old, donors were males and females 14–21 days old; in Series II recipients were 47 days old at hypophysectomy and donors were males 21–23 days old. In all animals except 2 of Series I there were pronounced gains in body weights, activation of the thyroids, structural repair of the adrenals and the reproductive organs, and fertility. The weights of the reproductive organs transcended those of normal controls. In 2 rats of Series I there were slower gains in body weights, and activation of the thyroids but no structural repair of the adrenals and reproductive organs. There were no "takes" when Sprague-Dawley instead of Long-Evans rats were used as donors.

THERE are but a few reports of transplantations of the pituitary gland into the region of the pituitary stalk and median eminence of the tuber cinereum, but the results obtained have been highly instructive. Greep (1) in 1936 in 1-stage operations made both auto- and homotransplantations of the pituitary gland into the evacuated hypophyseal capsules. He reports 73% of "takes" as judged by gain in body weight and by the functional activity of the reproductive system. Harris and Jacobsohn (2) in 1-stage operations hypophysectomized and then transplanted the hypophyses from the recipients' own young, into the empty pituitary capsules. In only 1 of 14 rats were the ovaries and adrenal glands found to be normal at autopsy. In one other, these organs were partly maintained; in the other 12 they were atrophic. Both Greep, and Harris and Jacobsohn used the parapharyngeal approach.

In a classical series of related experiments Harris and Jacobsohn (2), in 1-stage operations, hypophysectomized adult rats by the parapharyngeal approach, and then by the temporal approach, transplanted close to the median eminence, the hypophyses from the animals' own young. All 12 of the completely hypophysectomized rats had maintenance of the

Received June 30, 1960.

[1] This work was aided by a grant from the National Science Foundation.

[2] The assistance of Irene P. Smith is gratefully acknowledged.

ovaries, thyroids and adrenal glands. In a similarly operated series in which adult donors were used 5 of the 10 had estrous cycles; the other 5 had atrophic reproductive organs. The adrenal glands were maintained in most of the animals. In both of these series adult recipients were used, and consequently an influence on body weight could not be determined.

Nikitovitch-Winer and Everett (3) replanted pituitary autografts from the kidney into the region of the median eminence by the temporal approach. These replanted grafts acquired normal, or near normal function which had been lost while the grafts were on the kidney. Adults were used, so no effect on growth could be determined. Microsopic examination of the pituitary capsule made it possible to be certain of the completeness of the hypophysectomy, as in the transplantations of Harris and Jacobsohn to the median eminence.

In this paper the results obtained by the transplantation of the hypophysis into the region of the median eminence and pituitary stalk, by the parapharyngeal approach, are reported. In contrast to the temporal approach this is not a severe operation. Consequently, it could be employed in inherently delicate long-term hypophysectomized animals. It is evident that it is not possible to check the completeness of the hypophysectomy by a study of serial sections of the pituitary capsule. However the postponement of the transplantaions for a minimum period of at least a month, or as in the work here reported, for 2 or 5 months, permits the well-known hypophyseal syndrome to become well established, making it certain that the intracapsular portions of the hypophysis had been completely removed. The main external features of this syndrome are: no gain in body weight, usually a loss; reduction in muscle tonus resulting in a flaccid condition; and no evidence of testes within the scrotum, i.e., they can not be seen nor palpated. In females there is a narrowing of the vaginal orifice and a continuous diestrous type of vaginal smear.

EXPERIMENTAL PROCEDURE

The Long-Evans strain of rats was used. Although the colony has been propagated by random matings, a fairly close familial relationship exists, for it is a small colony. Sibs of the recipients were not used as donors.

Food consists of rat chow, lettuce, unground wheat and a daily supplementary feeding of cooked cereal to which dried skimmed milk and wheat germ were added.

The hypophysectomies were performed by the parapharyngeal approach. In order to increase the probability of complete removal of the anterior and posterior lobes, and also to facilitate later transplantation, a large opening was made through the basisphenoid. After removing the intracapsular component of the pituitary gland by suction with a large glass canula, a small curved one was inserted and the peripheral borders of the capsule were subjected to suction in an attempt to assure removal of any remaining fragment.

In the transplantation of the pituitary gland the parapharyngeal approach was again used. After exposure of the basisphenoid the hole through that bone made at hypophysectomy was located with a probe, the connective tissue covering it was removed and the

hole enlarged. The hole was never completely occluded although some growth of the bone had usually taken place at its periphery. The anterior part of the collapsed pituitary capsule and other soft tissues were gently torn to make a cavity for the transplant. The donor rat was then decapitated and its pituitary gland was aseptically removed and placed in the pocket which had been made. It was pushed in deeply with a small piece of masseter muscle, which served both to hold the transplant in place and to close the opening. In tearing the soft tissue to make a nest for the transplant some bleeding and a flow of cerebro-spinal fluid usually occurred. This operative technique permitted quite a precise placement of the transplanted gland near the median eminence and pituitary stalk. At the termination of the operation 4–5 cc of 5% dextrose in .85% saline, and Penicillin G (12,000 u.) were injected subcutaneously. Although long-time hypophysectomized rats are delicate, no deaths resulted from the transplantation operation. The animals were weighed daily at first and then twice each week.

Two series of experiments with male recipients and controls are here reported. The first series (Table 1) consists of 11 rats from our colony which were hypophysectomized between the ages of 38–42 days. Sixty days later each received a transplant of 1 pituitary gland. They were autopsied 77 days later. Two series of controls with posthypophysectomy periods of 60 days (Reference Controls), and 137 days (Terminal Controls), respectively, are also reported in the table, and a series of 10 hypophysectomized animals from our colony in which Sprague-Dawley rats were used as donors. For comparison of organ weights a series of normal unoperated rats autopsied at the approximate weights attained by the pituitaryless rats which received transplants, are listed.

In the second series (Table 2) data on 4 rats are reported, each of which received a single pituitary transplant 5 months after hypophysectomy. These were autopsied 70 days later. Data are given on hypophysectomized animals which received no transplants and were sacrificed after a posthypophysectomy period of 5 months, and also data on normal controls.

It is emphasized that the series of transplantations reported here are not selected from a larger group some of which were discarded. (In an earlier series of females a few of the transplants did not "take.") An occasional rat, however, was discarded during the posthypophysectomy pretransplantation period because it gained in weight or showed other signs of an incomplete hypophysectomy. The pituitary capsules of the hypophysectomized controls have been serially sectioned and found to contain no trace of the intracapsular components of the hypophysis.

RESULTS

SERIES I.—*Weight and Physical Condition.* After a posttransplantation period of 4–8 days a definite increase in weight of all rats of this series began, (Fig. 1). In 9 of the 11 this increase amounted to 11.2–17 gm. a week during the 77 days prior to autopsy. This is not as rapid an increase as in normal males of corresponding initial weights.

There was also an improvement in the physical condition of these 9 animals. Instead of being flaccid when handled as is characteristic of rats which have been pituitaryless for some time, muscle tonus seemed equal to that of normal rats. Also their pelage and appetite seemed normal.

Two of the 11 rats receiving transplants showed characteristics which clearly separated them from the other 9, (Table 1, Fig. 1). Although they increased in weight, and this increase began as soon after the transplantation as in the other 9 rats, it was markedly less, amounting to 3–4 gm. per

week. In spite of this slow rate of gain their weights at autopsy exceeded their pretransplantation weights by 34 gm. and 44 gm., respectively, Improvement in their physical condition and pelage was not clearly evident. At autopsy no unusual deposits of fat, which could account for their gain in weight, were found.

Skeletal Growth. The proximal epiphyseal discs of the tibiae of both the 60-day and 137-day hypophysectomized controls have structural features characteristic of cessation of growth (Fig. 6). The cartilage cells are small and the intervening cartilage between the cell columns is greater than in growing animals. Erosion of the cell columns had ceased as is evidenced by the formation of a thin layer of bone over much of the medullary surface of the disc. Medullary trabeculae are almost completely absent except at the periphery of the disc. The structural features are similar to those described in hypophysectomized female rats, (4, and earlier papers).

The epiphyseal discs (Figs. 2, 3, 4, 5) of the hypophysectomized rats which received pituitary transplants are strikingly different from the pituitaryless controls described above. No layer of bone is present on the medullary surface of the disc. The lacunae in the vesicular zone of the discs are large and some open into the medullary cavity. Many bony trabeculae with central cores of calcified cartilage extend into the medullary cavity. The structural features are characteristic of cartilage growth and its replacement by bone.

It was stated earlier that 2 of the rats which received transplants gained in weight more slowly than the other 9 animals. The epiphyseal discs of their tibiae (Figs. 3, 4), although not showing characteristics of as rapid growth as the other 9 rats, nevertheless differ markedly from the pituitaryless controls. There is no layer of bone on the medullary side of the disc. The lacunae of the vesicular zone are large and some open into the medullary cavity and contain vascular sprouts. Bony trabeculae with cores of cartilage are present. As shown in Figure 1 a gain in weight was continuing at the time of autopsy.

Testes, Epididymides, Mating. The testes of the group of 9 rats which grew more rapidly became sufficiently enlarged within 14–18 days after the transplantation to be easily palpated. This was only 9–13 days after an increase in body weight began. The weights of the testes of these 9 animals exceed the weights of the testes of normal unoperated controls of corresponding body weights (Table 1). They exceed the weights of the testes of the hypophysectomized controls autopsied 60 days after the removal of the pituitary gland (Hypx R C) by 16 times, showing the great growth that had taken place. The testes of these 9 rats are normal in structure (Fig. 10). All the seminiferous tubules have the various stages of spermatogenesis and many contain spermatids or sperm.

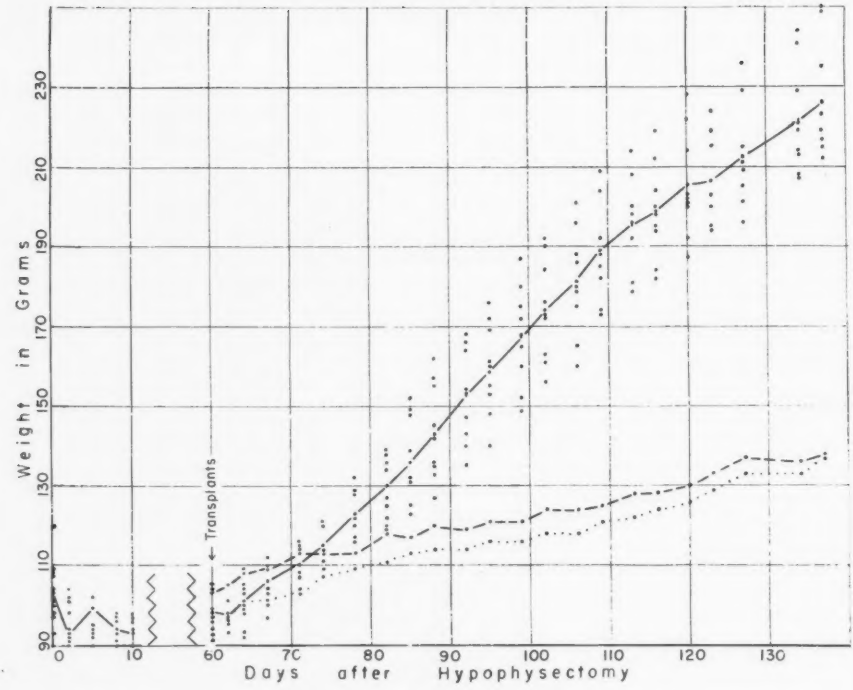


FIG. 1. Growth data for the 11 rats of Series I which received transplants of hypophyses from the Long-Evan's strain (see Table 1).

FIGS. 2–6. Sagittal sections of proximal tibial discs of male rats. Delafield's hematoxylin and eosin. ×180.

FIG. 2. Forty days old at hypophysectomy (109 gm.). Sixty days later (93 gm.) given 1 transplant of an A.P. from a 21 day-old male. Autopsy 77 days later (212 gm.). See Table 1.

FIG. 3. Littermate of the above. Forty days old at hypophysectomy (120 gm.). Sixty days later (104 gm.) given 1 transplant of an A.P. from a 21 day-old male. Autopsy 77 days later (138 gm.). Had an active thyroid (Fig. 9) and atropic adrenal glands and testes.

FIG. 4. From a littermate with a similar history.

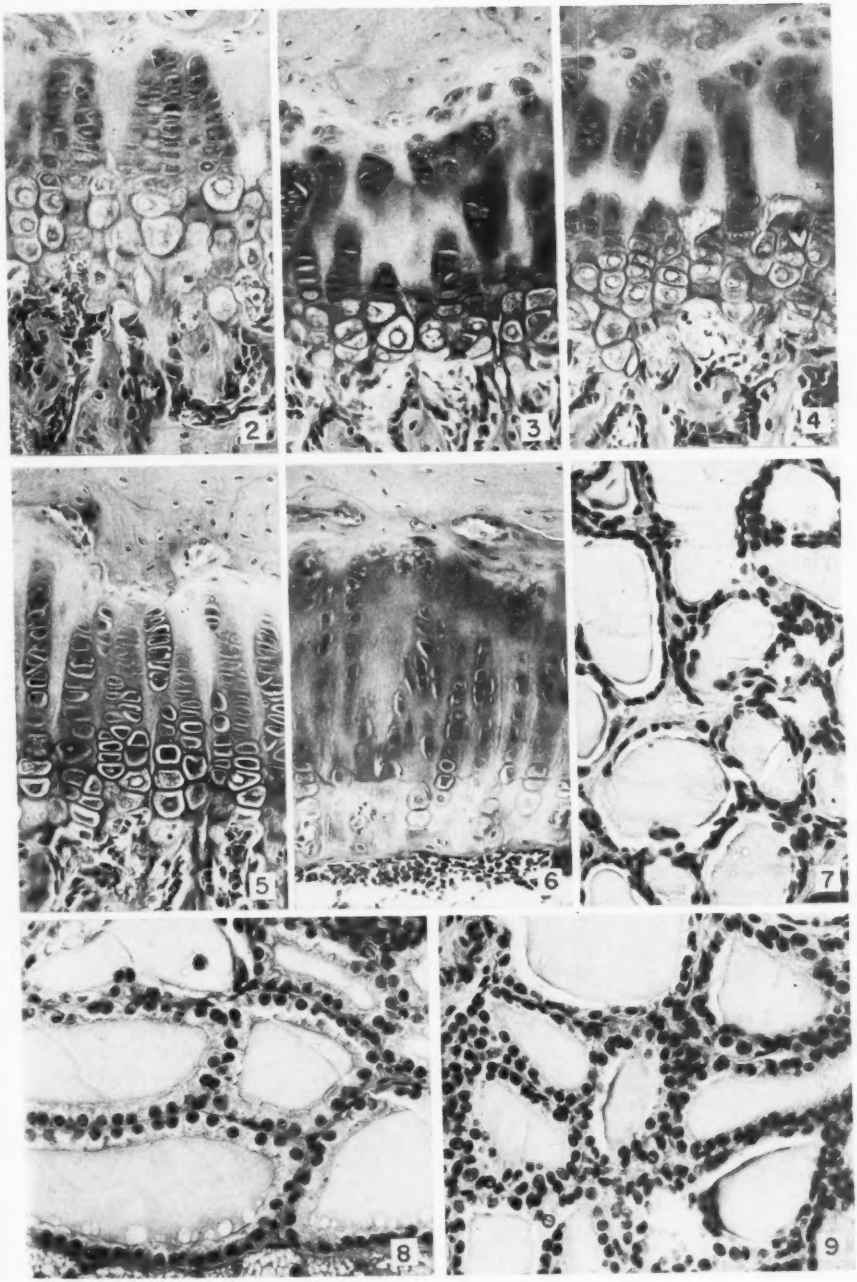
FIG. 5. Forty-seven days old at hypophysectomy (137 gm.). Five months later (12[illegible] gm.) given 1 A.P. transplant from a 21 day-old male. Autopsied 75 days later (200 gm.).

FIG. 6. Terminal control. Hypophysectomized when 41 days old (102 gm.) and autopsied 150 days later (96 gm.).

FIG. 7. Thyroid of an hypophysectomized reference control 60 days after the ablation of the pituitary gland. ×315.

FIG. 8. Thyroid of rat described in legend of Fig. 2. ×315.

FIG. 9. Thyroid of rat described in legend of Fig. 3. ×315.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The weights of the testes of the 2 rats which grew more slowly than the other 9 are within the range of the hypophysectomized controls (Table 1). Structurally they show no repair. (Fig. 14).

The epididymides of the 9 animals which grew more rapidly after receiving transplants of pituitary glands have a relative increase in weight that is even greater than that of the testes. They are nearly double the weights of the epididymides of normal animals of corresponding body weights. A study of sections reveals large numbers of sperm. The epididymides of the 2 rats which grew more slowly show no repair.

Each of the 9 rats of the more rapidly growing group was tested one or more times for fertility by placing a female in proestrus with it. Each sired from 1–3 normal litters. The earliest that a female was placed with one of these males was 46 days following transplantation. The pair mated and normal young were born.

The Adrenal Glands. The weight and structure of the adrenal glands separates the 11 rats which received transplants of the hypophysis into 2 groups as definitely as does body growth and the condition of the reproductive system. The weights of the adrenal glands of the 9 rats which grew more rapidly increased markedly. However, they did not quite attain a mean weight equal to that of normal controls of corresponding body weight. They also have more variation in weight. In 4 of them the adrenal weights are within the range of the normal controls. In 1 of these 4 the adrenal weight nearly equals that of the heaviest in the controls. The weights of the adrenal glands are not correlated with their body weights. Their variation contrasts with the uniformity of the weights of the adrenals of the hypophysectomized controls.

Structurally the adrenal glands of these 9 rats appear to be normal.

FIG. 10. Testis of animal described in legend of Fig. 2. ×90. See Table 1.

FIG. 11. Testis of animal described in legend of Fig. 5. ×90. See Table 2.

FIG. 12. Testis of a littermate with a history similar to that of the above animal. ×315.

FIG. 13. Testis of hypophysectomized terminal control. Autopsied 37 days after the removal of the pituitary gland. ×315.

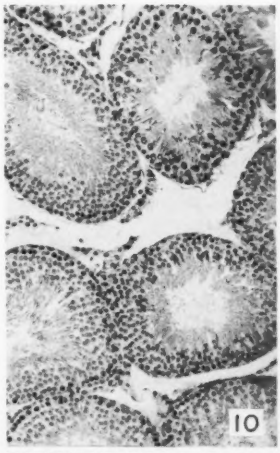
FIG. 14. Testis of animal described in the legend of Fig. 3. ×315.

FIG. 15. Cortex of adrenal gland of littermate of animal described in Fig. 5. H & E stain. ×123.

FIG. 16. Cortex of adrenal gland of an animal given a transplant of 1 hypophysis from a 14 day old male after a posthypophysectomy period of 60 days (Table 1, Fig. 1), and autopised 77 days later. Oil red O stain. ×123.

FIG. 17. Adrenal cortex and subjacent medulla of animal described in legend of Fig. 6. The limit of the cortex is marked with an arrow. H & E stain. ×123.

FIG. 18. Adrenal cortex and subjacent medulla of an 137-day hypophysectomized terminal control. (Table 1) oil red O stain. ×123.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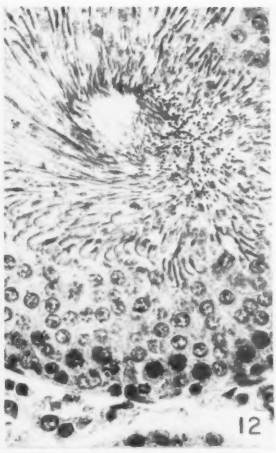
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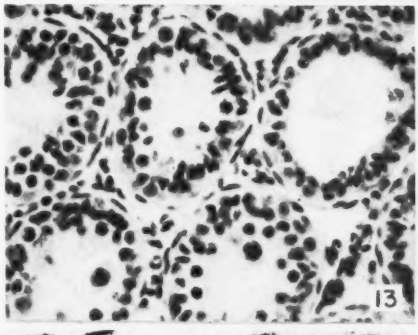
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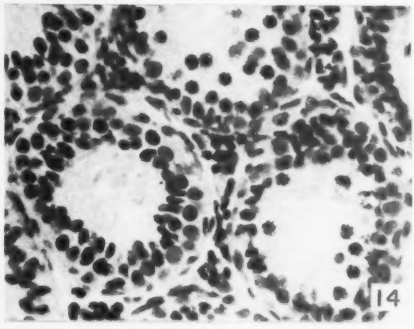
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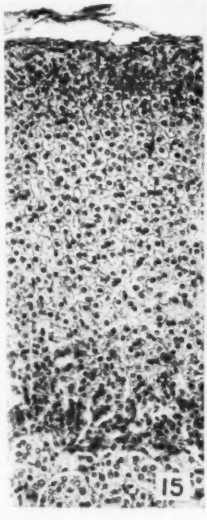
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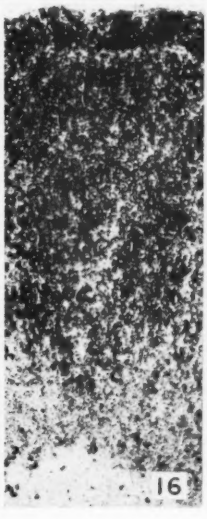
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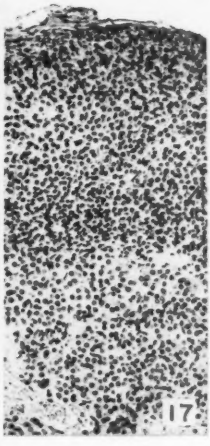
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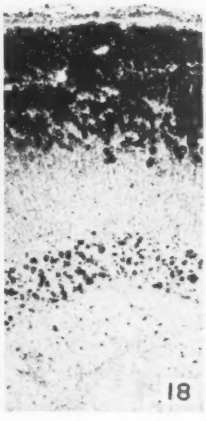
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TABLE 1. DATA ON MALE RATS EACH OF WHICH WAS GIVEN A SINGLE TRANSPLANT OF ONE HYPOPHYSIS AFTER A POSTHYPOPHYSECTOMY PERIOD OF 60 DAYS

Data also given on hypophysectomized and normal controls. All animals were 38–42 days old at the beginning of the experiment. *Hypx+Trpl*—Hypophysectomized rats which received pituitary transplants from Long-Evans rats. Each of 4 of these rats received a whole hypophysis from a 17 day old female, 4 each received a whole hypophysis from a 14 day old male and 3 received anterior hypophyses from 21 day old males. The recipients were autopsied 77 days after the transplantation (137 days after hypophysectomy). *Hypx T C*—Hypophysectomized terminal controls. They were autopsied 137 days after hypophysectomy. *Hypx R C*—Hypophysectomized reference controls. They were autopsied 60 days after hypophysectomy. *Nor T C*—Normal terminal controls. Unoperated rats which were autopsied at the approximate weights attained by 9 of the 11 hypophysectomized rats which received pituitary transplants from Long-Evans rats. *Hypx+S-D Trpl*—Each of these rats received a transplant of a pituitary gland from the Sprague-Dawley strain of rats.

No. of rats	Type	Body weight, mean and range (gm.)		Autopsy, mean and range (gm.)			
		At Hypx	At Trpl	Body wt.	Testes	Epid.	Adrs.
9	Hypx+Trpl	101 (93–109)	98 (91–105)	227 (212–252)	2.74 (2.45 –2.96)	.75 (.61 – .88)	.030 (.022–.040)
2	Hypx+Trpl	108, 120	93, 104	137, 138	.17, .21	.057, .087	.011, .010
4	Hypx T C	108 (102–113)		101.5 (96–107)	.166 (.153– .189)	.037 (.028–.046)	.010 (.008–.011)
9	Hypx R C	124 (119–128)		119 (112–115)	.170 (.146– .230)	.049 (.027–.098)	.010 (.008–.011)
9	Nor T C			227 (215–249)	2.27 (1.97 –2.45)	.38 (.26 – .54)	.035 (.031–.041)
10	Hypx+S-D Trpl	111 (103–128)	106 (97–118)	111 (99–127)	.15 (.106– .176)	.038 (.026–.048)	.010 (.008–.012)

The nuclei of the fascicular zone are widely separated and, after fat solvents, the cytoplasm is vacuolated (Fig. 15). Frozen sections of their adrenal glands fixed in formalin and stainned with a fat dye (oil red O) show a normal distribution of fat (Fig. 16). Their structure is in striking contrast with that of the adrenal glands of the hypophysectomized controls (Figs. 17, 18).

The weights of the adrenal glands of the 2 hypophysectomized rats which gained slowly in body weight after receiving pituitary transplants are within the range of the pituitaryless controls and they have the structural features of the adrenal glands of the long-term hypophysectomized controls.

Thyroids. Although the structure of the thyroids in rats does not portray the functional state as clearly as in some other laboratory animals, nevertheless, the structural features are distinct enough in the series here reported to enable conclusions to be drawn.

The thyroid follicles of the hypophysectomized reference and terminal controls have a uniformly flat epithelium characteristic of animals which have been pituitaryless for extended periods (Fig. 7). In contrast, the thyroids of the 9 more rapidly growing hypophysectomized rats which received transplants have areas in which the follicles are lined by cuboidal epithelium (Fig. 8). The structure of these follicles clearly indicates activation.

The follicles in other areas have a flat epithelium. Structural evidence thus indicates a spotty response to the thyreotrophic output of the transplanted pituitary gland.

We had expected to find that the 2 rats which grew slowly after receiving pituitary transplants would have no structural features indicating activation of the thyroids and thus would correspond to the condition obtaining in their adrenal glands and testes. This proved not to be the case. The follicular epithelium in areas of the thyroids of these 2 rats is cuboidal (Fig. 9). The structural response is not as pronounced as in the more rapidly growing group. It seems adequate, however, to indicate a response paralleling skeletal growth.

Sprague-Dawley Strain of Rats as Donors. The high percentage of "takes" in the transplantation of hypophyses from rats of our colony suggested using another strain (Sprague-Dawley) as donors in order to determine if there is a strain specificity. As shown in Table 1, 10 rats from our colony were given transplants of the hypophyses from the S-D strain. The posthypophysectomy period at the time of the transplantation in 2 of them was 54 days, with 6 day old donors; in 4 it was 57 days with donors 18 days of age; and in 4 it was 63 days, donors being 22 days old.

In all recipients there was some gain in weight after the transplantation. In 7 of the 10 this was sufficient to carry them above their prehypophysectomy weight by 2–11 gm. This gain was followed by a recession so that the mean of the body weights at autopsy, 38 days later, was the same as it was when their pituitary glands were ablated. The weights of the testes, epididymides and adrenal glands are within the range of the pituitaryless controls.

Although the gain in body weight is small it seems sufficient to indicate a temporary secretion by the transplant. It appears improbable that it could be caused by a release of stored hormone in the donor glands because of their small size especially with donors only 6 days old.

Except for the temporary increase in body weight, the results from the transplants of the hypophyses from Sprague-Dawley rats are negative.

The temporary and essentially negative effects provide an excellent control for our other transplantations. They reinforce our data in showing that the effectiveness of transplants from animals of our colony as donors is due to the secretory activity of the transplant itself and not to an activation of some unremoved hypophyseal frgament by the operative procedure of the transplantation.

SERIES II. This series is composed of 4 rats which were given pituitary transplants after a posthypophysectomy period of 5 months, 4 hypophysectomized reference controls and 10 unoperated controls. Although the

TABLE 2. DATA ON 4 MALE RATS EACH OF WHICH WAS GIVEN A SINGLE TRANSPLANT OF 1 HYPOPHYSIS AFTER A POSTHYPOPHYSECTOMY PERIOD OF 150 DAYS

Data are also given on hypophysectomized and normal controls. *Hypx+Trpl*—Hypophysectomized rats which received transplants. The rats were 47 days old at the time of hypophysectomy. Each rat lost weight during the 150 day posthypophysectomy period prior to the transplantation, the loss ranging from 9–27 gm. Donors were males ranging in age from 21–23 days. *Hypx R C*—Hypophysectomized reference controls. These received no transplants and were autopsied 150 days after the removal of the pituitary gland. *Nor T C*—Normal terminal controls. Normal unoperated rats.

No. of rats	Type	Body weight, mean and range (gm.)		Autopsy, mean and range (gm.)			
		At Hypx	At Trpl	Body wt.	R testis[1]	R Epid[1]	2 Adrs.
4	Hypx+Trpl	137 (130–142)	120 (110–133)	210 (200–229)	1.38 (1.28 –1.45)	.376 (.361–.394)	.025 (.022–.028)
4	Hypx R C	122 (109–140)		112 (97–127)	.084 (.074– .098)	.021 (.016–.026)	.010 (.009–.011)
10	Nor T C			215 (207–223)	1.14 (1.05 –1.20)	.187 (.138–.254)	.035 (.031–.042)

[1] The left testis and left epididymis of each of the hypophysectomized rats given pituitary transplants were removed 20–40 days after the transplantation. In order that the data may more nearly correspond, the weights of the right testes and right epididymides are given for the hypophysectomized controls and the normal terminal controls.

characteristic features of hypophyseopriva were more pronounced in these rats than in those given transplants after a pituitaryless period of 60 days, none succumbed to the operation.

Weight and Skeletal Growth. After a posttransplantation period ranging from 4–6 days a gain in weight occurred which continued until autopsy. Counting from the day when the gain first became unequivical the increment ranged from 1.1–1.6 gm. a day. Although this seemed spectacular compared to the long period of growth stasis prior to transplantation, it is only $\frac{1}{4}$–$\frac{1}{3}$ the rate of increase of growth in normal rats of corresponding size in our colony.

Tibiae. The epiphyseal discs of the heads of the tibiae of the animals given transplants show the structural features characteristic of bone growth (Fig. 5). The columns of cartilage cells are long and the lacunae in the zone of calcification are large and contain robust cartilage cells. Many lucunae open into the medullary cavity and contain erythrocytes indicating that vascular sprouts had penetrated them. Bony trabeculae with central cores of calcified cartilage extending into the medullary cavity are frequent.

Reproductive System and Reproductive Behavior. The testes of each of the hypophysectomized rats given pituitary transplants became sufficiently large and firm to be readily palpated by 10 days after the operation. Between days 20 and 40 following transplantation each rat was unilaterally castrated in order to secure evidence on the rate of repair of the testes.

The remaining testis of each rat at autopsy 75 days after the hypophyseal transplantation weighed more than a single testis of any of the normal controls (Table 2), there being no overlapping in the weights be-

tween the 2 groups. Each testis exceeded in weight a single one of the hypophysectomized reference controls by over 15 times. Structurally the seminiferous tubules of the rats given transplants are normal (Fig. 11). An occasional tubule, although enlarged, did not undergo repair. All stages of spermatogenesis and many sperm are present (Figs. 11, 12). The interstitial cells of Leydig, also, are normal. It is realized that the weights of the remaining testes of the rats receiving transplants may have been enhanced by the unilateral castration, although the latter did not occur until near the mid-point between the transplantation and autopsy.

The weights of the epididymides of the hypophysectomized animals receiving transplants as compared to normal controls of corresponding body weights are even greater than in the case of the testes. The epididymides are normal in structure and contain numerous sperm.

The mating reaction of the hypophysectomized males given transplants was normal. Between 50–60 days after the males received transplants a normal female in proestrus was placed with each one. The males displayed marked libido and each of them sired one or more normal litters.

Adrenal Glands. The adrenal glands of the hypophysectomized rats receiving pituitary transplants, although increasing markedly in weight did not attain the mean weight of normal controls of corresponding body weight (Table 2). Structurally, as in Series I, there was an enlargement of the cells of the fascicular and reticular zones. The distribution of fat in frozen sections stained with oil red O was normal.

DISCUSSION

The percentage of "takes" in the series of pituitary transplantations in male recipients reported here, is higher than in a larger series of females (5). Two factors may play a role in this, 1) an improvement in operative technique, 2) a closer relationship of recipients and donors due to continued inbreeding. We are inclined to believe that the latter is more important. The animals of our colony came from the parent stock in 1927. During the past 3 years there has been a high degree of inbreeding. We find that Long-Evans rats purchased from a colony reared independently of ours gives a very low percentage of "takes" when used as donors. There were no "takes" with Sprague-Dawley rats as donors although there appeared to be a brief period of secretion by the transplant.

The early gain in weight (4–6 days) following the transplantation of the pituitary gland indicates a rapid vascularization of the transplant. Intravascular injections with India ink or Berlin blue show that the transplants have been richly vascularized.

Although our long-term hypophysectomized males had a surprisingly prompt response in body weight to the transplantation of the hypophysis, the later gain, as in the more acute experiments of Greep (1), was not as

rapid as in the normal males of corresponding size. This slower rate of increase is reflected in the structure of the proximal tibial discs. We have not found in the literature a description of the effects of ageing on skeletal growth in male rats except in young and very old ones. The sequence of changes with age in normal females of the Long-Evans strain of rats, however, has been reported (6). The tibial cartilage discs in our series of hypophysectomized males with pituitary transplants correspond closely to the description and figures of normal females 74 days old. At this age normal females have a decreased rate of growth.

The adrenal glands in hypophysectomized males receiving transplants did not quite attain the weights in normal males of corresponding body weights.[3] Structurally the adrenal glands were indistinguishable from those of normal males. Harris and Jacobsohn (2) report in their transplantations of the hypophysis near the median eminence that most of the adrenal glands were of normal weight.

The thyroids, although having areas of pronounced activation, did not quite attain a normal structure for there were areas with atrophic epithelium. Apparently the transplanted pituitary glands did not release sufficient TH to activate the entire gland.

Except in the 2 more slowly growing hypophysectomized males with transplants the testes and epididymides stand out as giving a much greater response than the other organs. The increase in these organs was great enough to make them transcend the weights in the normal controls. The long period of atrophy did not affect the capacity of the seminiferous tubules to respond to gonadotrophic hormone.

The 2 hypophysectomized rats which grew more slowly than the others after receiving transplants and had activation of the epiphyseal discs and thyroids but no repair of the adrenal cortex or testes, present a puzzling combination. The activation of the thyroid would enhance the response to somatotrophic hormone because of a synergistic action (7, 8, 9, 10, 11). The failure of a response of the testes in these 2 rats can hardly be attributed to a higher threshold judging from the response of these organs in the other animals. It is possible that an analysis of the blood supply and cell types

[3] Throughout this paper the weights of organs in the hypophysectomized rats receiving pituitary transplants have been compared with weights in normal controls of corresponding body weights. This seems the most valid comparison to make. The weights in normal animals of corresponding age obviously cannot be compared with the hypophysectomized ones given transplants. The latter, so far as an increase in body and organ weights is concerned, had periods of 2 or 5 months dropped out of their lives before the transplantations were done. During this period the epiphyseal discs and the organs discussed here underwent a pronounced atrophy. This atrophy had first to be repaired by the secretions of the transplants before the structures attained their prehypophysectomy condition. This involved an additional period of time. Percentages of organ weights to body weights have been computed but they do not add to the comparison of medians and ranges.

may aid in an explanation. This analysis will be presented in another communication.

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THE PHYSIOLOGICAL FRACTIONATION OF PITUITARY GONADOTROPIC FACTORS CORRELATED WITH CYTOLOGICAL CHANGES[1]

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ABSTRACT

Removal of estrogen or androgen influence by gonadectomy and the controlled administration of gonadal steroids resulted in regulation of the concentration of follicle stimulating (FSH) and luteinizing (LH) factors in pituitary glands of young adult rats. These physiological changes were correlated with cytological findings.

Gonadectomy in both sexes results in a marked increase in both LH and FSH in the pituitary as well as a corresponding increase in the red and purple (PAS-Methyl blue staining) pituitary gonadotropes. The administration of the equivalent of 3 μg. of estradiol benzoate per day over a 45-day period stimulates depletion of LH. FSH is also markedly decreased. The cytological picture shows very few red but some purple gonadotropes. On the other hand, the administration of the equivalent of 3 mg. of testosterone propionate per day over a 45-day period also depletes the LH but the FSH content remains high and the correlated cytological findings show a corresponding decrease in the red gonadotropes, but with little effect on the purple ones. These findings show that FSH and LH can be differentiated by physiological means and support the concept that the red gonadotropic cells are related to LH production or storage, and the purple ones to FSH production or storage.

THE chemical fractionation of the pituitary gonadotropic complex into separate follicle stimulating (FSH) and luteinizing (LH) has been accomplished with varying degrees of success (1–7). The present report deals with physiological methods of obtaining FSH essentially free from LH, altering the level of LH and correlating the pituitary gonadotropin content with cytological changes in the anterior lobe of the pituitary gland. Previous reports have shown that both estrogens and androgens stimulate liberation of pituitary LH (8–10).

METHODS AND PROCEDURES

Alterations in FSH and LH concentrations in the pituitary glands of normal young adult male and female Sprague-Dawley or Holtzman rats were determined under the

Received July 29, 1960.

[1] Supported in part by a grant from the Merrick Foundation, Ardmore, Oklahoma, and by a contract between the Office of Naval Research, Department of the Navy, and the University of Miami, NR 101–140.

following conditions: 1) normal, untreated; 2) gonadectomized, untreated; 3) gonadectomized, treated for 45 days with estradiol benzoate; and 4) gonadectomized, treated for 45 days with testosterone propionate.

An interval of at least two months was allowed after removal of the gonads, to permit changes in the pituitary gland to stabilize before estrogen or androgen was administered. The gonadal steroids were injected subcutaneously, every other day, in 0.05 ml. of purified sesame oil. This is the amount previously determined to be absorbed in a 48 to 72 hour period.

After administration of the steroids on alternate days over a period of 45 days, pituitaries were removed, and FSH and LH activities evaluated and cytological studies made. The pituitary glands were prepared for assay by placing them into acetone immediately upon removal, changing the acetone twice, permitting the pituitaries to airdry, placing in vials and storing in the dark. In this form the gonadotropic factors remain stable indefinitely.

At the time of assay, the dried pituitary glands were weighed, and then ground individually in a special small glass "pestle and mortar" described previously (11). The powdered material was suspended in saline or distilled water. Test animals were either 21 days old normal, or immature hypophysectomized, female Sprague-Dawley or Holtzman rats. Each was injected with 0.5 ml. of pituitary suspension, twice daily for 3 days; then autopsied 120 hours after the first injection.

At the time of autopsy the ovaries were examined grossly, with the aid of a strong indirect light, to observe degree of follicular stimulation and presence or absence of lutein tissue. Ovaries in which there was any question concerning luteinization were prepared for microscopic examination; approximately one-half of the ovaries were thus examined histologically. However, the experience gained from the examination of many ovaries has made it possible to correlate gross observations closely with microscopic findings.

Pituitary glands to be studied microscopically were fixed in buffered 10% formol-saline, and stained with the modified PAS-Methyl blue stain described by Rennels (12) and Hildebrand, Rennels and Finerty (13).

RESULTS

I. Pituitary FSH and LH Content

Alterations in FSH and/or LH levels, as well as in morphology, occurred in the pituitary glands of both males and females following gonadectomy, and after gonadectomy followed by administration of estradiol benzoate or testosterone propionate.

A. Pituitary glands of female rats. Data on the females are presented in Table 1. The extent and type of ovarian response resulting from the administration of normal adult female rat pituitaries, whether tested in normal or hypophysectomized immature recipients, were similar. The ovaries of all the recipients contained lutein tissue. In addition, all of the pituitaries from animals in which the estrogen influence had been removed by ovariectomy two months previously were found to have a marked increase in both LH and FSH over the pituitaries of normal rats. Thus 4–6 glands from unspayed animals, weighing dry 11.5 to 13.9 mg., induced less ovarian stimulation than did one-half of a pituitary (1.5 mg. dry weight) of a spayed animal. The ovaries of all of the 50 recipients used to assay the pituitaries of the spayed donors were heavily luteinized.

TABLE 1. QUALITATIVE CHANGES IN THE FSH:LH CONCENTRATIONS OF FEMALE RAT PITUITARIES FOLLOWING 1) SPAYING, 2) SPAYING AND ESTROGEN ADMINISTRATION, AND 3) SPAYING AND ANDROGEN ADMINISTRATION AS REFLECTED IN THE OVARIES OF NORMAL AND HYPOPHYSECTOMIZED IMMATURE RATS

Adult female donor pituitaries		Immature female recipients					
				Ovarian weights		Number having	
Type	No.	No.	Av. total amt. pit. tis. inj. per rat (mg.)	Range (mg.)	Average (mg.)	Foll. only	C.L.
		Normal recipients					
Normal	30	6	11.5	16– 59	34	—	6
Spayed—2 mo.	18	18	2.9	28– 93	73	—	18
	8	16	1.4	19–109	52	—	16
Spayed—2 mo. 3 μg. E.B./day/45 da.	26	13	9.8	21– 73	41	10	3
Spayed—2 mo. 3 mg. T.P./day/45 da.	14	28	1.4	22– 71	36	23	5
		Hypophysectomized recipients					
Normal	24	4	13.9	19– 34	29	—	4
Spayed—2 mo.	8	16	1.5	28– 73	46	—	16
Spayed—2 mo. 3 μg. E.B./day/45 da.	16	8	9.9	27– 41	32	8	—
Spayed—2 mo. 3 mg. T.P./day/45 da.	8	16	1.4	26– 48	31	15	1

E.B. = estradiol benzoate.
T.P. = testosterone propionate.
C.L. = Corpora lutea.

Similar donor adult female rats that had been spayed two months previously were injected with 6 μg of estradiol benzoate every other day for a period of 45 days. Two pituitary glands from animals so treated, and weighing on the average 9.8 mg. after acetone desiccation, were administered to each of 13 normal and 8 hypophysectomized recipients. The ovaries of 10 of the normal recipients, and of all of the hypophysectomized recipients, contained follicles only, indicating absence or low levels of LH. Other animals have received as little as 1 μg. estradiol benzoate per day for the same period of time, with similar results.

Testosterone propionate in dosages equivalent to 3 mg. per day was administered over a period of 45 days to 22 young adult donor animals, beginning two months after ovariectomy. The pituitaries of these animals were assayed in normal and hypophysectomized immature rats. The ovaries of 23 of the 28 normal test rats, and of 15 of the 16 hypophysectomized animals, showed follicular development only. Luteinization in varying degrees was found in the ovaries of only six of the 44 test animals.

Although androgen and estrogen both deplete the pituitary of LH, they

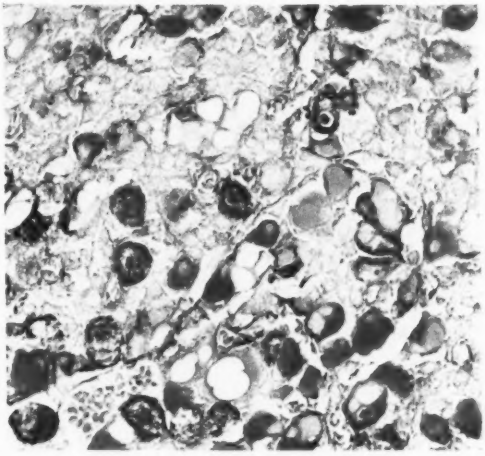

FIG. 1. Three months after gonadectomy. Showing increased number of both red and purple gonadotropes.

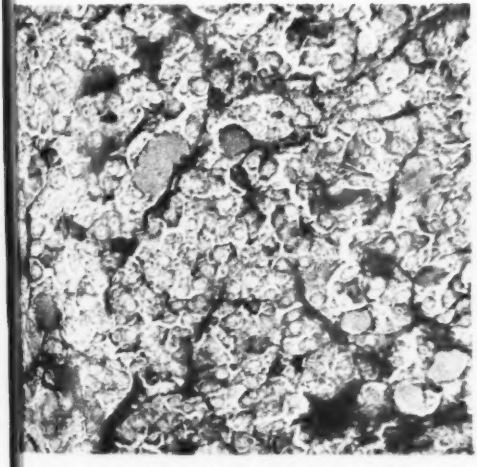

FIG. 2. Gonadectomized three months, then injected with estradiol benzoate, 3 μg./day/45 days. The red cells are depleted and a few scattered purple cells remain.

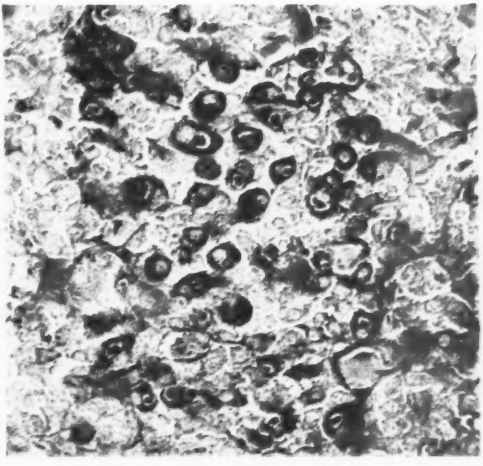

FIG. 3. Gonadectomized three months, then injected with testosterone propionate, 3 mg./day/45 days. Red cells absent while the number of purple cells, which increased following gonadectomy, remained high.

FIGS. 1–3. Histological appearance of the anterior pituitary glands of adult female rats. PAS-methyl blue stain, ×342.

TABLE 2. QUALITATIVE CHANGES IN THE FSH:LH CONCENTRATIONS OF MALE RAT PITUITARIES FOLLOWING 1) CASTRATION, 2) CASTRATION AND ESTROGEN ADMINISTRATION, AND 3) CASTRATION AND ANDROGEN ADMINISTRATION AS REFLECTED IN THE OVARIES OF NORMAL AND HYPOPHYSECTOMIZED IMMATURE RATS

Adult male donor pituitaries		Immature female recipients					
		No.	Av. total amt. pit. tis. inj. per rat (mg.)	Ovarian weights		Number having	
Type	No.			Range (mg.)	Average (mg.)	Foll. only	C.L.
		Normal recipients					
Normal adult	35	14	5.5	21–57	31	12	2
Castrated 2 mo.	11	22	2.1	36–97	67	—	22
	6	24	1.0	17–51	39	—	24
Castrated 2 mo. 3 μg. E.B./day/45 da.	32	16	9.6	25–61	47	14	2
Castrated 2 mo. 3 mg. T.P./day/45 da.	14	28	1.6	32–83	54	24	4
		Hypophysectomized recipients					
Normal adult	44	11	8.8	28–46	36	10	1
Castrated 2 mo.	9	18	2.0	36–57	46	—	18
Castrated 2 mo. 3 μg. E.B./day/45 da.	22	11	9.8	15–47	34	11	—
Castrated 2 mo. 3 mg. T.P./day/45 da.	7	14	1.7	28–53	41	13	1

E.B. = estradiol benzoate.
T.P. = testosterone propionate.

differ in their effects on pituitary levels of FSH. Estradiol benzoate decreases the concentration of FSH as well as of LH, whereas testosterone propionate, at the dose-level used, does not affect FSH to any appreciable extent.

B. Pituitary glands of male rats. Table 2 shows the response in normal and hypophysectomized recipients, to the administration of various types of young adult male pituitaries. Contrary to the gonadotropic activities in intact female rat pituitaries, those of normal males stimulated corpora lutea in the ovaries of only three of the 25 test animals. This observation confirms previous reports that the pituitary gonadotropin content of normal male rats is primarily FSH (10, 11).

On the other hand, removal of the testicular influence by castration alters the pituitary gonadotropic complex from predominance of FSH to high levels of LH. Heavy luteinization was seen in the ovaries of all 64 recipients, assaying the pituitaries of castrated males (Table 2). Also, one fourth of a pituitary gland from a castrated male rat (equal to one mg. of dried pituitary powder) was found to increase the weight of the ovaries of

assay animals as much as did two and a half pituitaries from intact animals (5.5 mg. dried weight).

Similar adult males, that had been castrated two months previously, were injected with 6 μg of estradiol benzoate every other day over a period of 45 days. In the ovaries of the recipients testing these pituitaries, only two of the 16 normal and none of the hypophysectomized test animals, contained lutein tissue.

That androgen, like estrogen, stimulates release of LH was demonstrated by a group of 21 castrated male rats which received 6 mg. of testosterone propionate every other day over a period of 45 days. The pituitaries of these donor animals were administered to normal and hypophysectomized recipients. Corpora lutea were found in the ovaries of only four of the 28 normal test animals; and of only one of the 14 hypophysectomized recipients.

II. Cytological Changes

It is generally known that estrogens and androgens may affect the pituitary gland not only functionally but also structurally. A relationship between cytological structure and pituitary gonadotropic activity can today hardly be questioned.

Reports of Purves and Griesbach (14) suggest that peripherally situated basophil gonadotropes in the pituitary, distinguished by coarse, intensely PAS staining cytoplasmic granulation due to glycoprotein, are responsible for secretion of FSH. Centrally located basophil gonadotropes, having a finer granulation and less intense glycoprotein reaction, are considered by them to be the source of LH. These conclusions appear to be supported by electron microscope studies by Farquhar and Rinehart (15). But Hildebrand *et al.* (13), although accepting the existence of two types of basophil gonadotropes, consider the peripheral ones to be LH secretors and the central ones to be the FSH producers. The latter study utilized the PAS-methyl blue technique (12) which differentiated "red" and "purple" gonadotropes.

In the present study, gonadectomy was found to result in an extensive increase in number and size of red and purple basophils, with concomitant granulation and vacuolization in both peripheral and central areas (Fig. 1). These changes were related to increases in both FSH and LH, as demonstrated in test animals. Following administration of estradiol benzoate, cytological study revealed a decreased number of basophils in the angles adjacent to the pars intermedia as well as in the central zone, with loss of vacuolation and granulation, and moderate increase in chromophobes (Fig. 2). This cytological picture correlated well with the decreased FSH and LH content, and tended to be similar to that seen in intact, uninjected control rats.

Testosterone propionate, in dosages of 3 mg. daily, reduced the number

TABLE 3. CORRELATION OF CYTOLOGICAL FINDINGS WITH PHYSIOLOGICAL RESPONSE IN PITUITARIES OF NORMAL, GONADECTOMIZED AND TREATED ADULT MALE AND FEMALE RATS

Type	Cytological findings	Physiological results
Normal female	4–6% basophils, both red and purple type gonadotropes	FSH and LH present (weak)
Normal male	8–10% basophils, preponderance of purple type	More gonadotropic activity—mostly FSH
Gonadecomized (both sexes)	Extensive increase in both red and purple types	Increase in LH and FSH (greater LH)
Gonadectomized (both sexes), treated with estradiol benzoate	Very few red—some purple cells remaining	No LH. Marked decrease in FSH
Gonadectomized (both sexes), treated with testosterone propionate	Depletion of red cells while purple remain	No LH. FSH concentration remains high

of red gonadotropes so that it was difficult to find any in a representative microscopic section. However, an abnormally high number of purple gonadotropes remained (Fig. 3). The number of purple cells is somewhat similar to that seen in early castration, described by Hildebrand *et al.* (13).

Decrease in total gonadotropin content of the pituitary glands, after estradiol administration, was accompanied by decreases in both purple and red gonadotropic cells. Testosterone administration, on the other hand, decreased the number of red gonadotropes but had little effect on the purple ones. These observations, coupled with the animal assay data showing a decrease in LH but little change in FSH, support the concept that the red gonadotropes are related to LH, and the purple ones, to production or storage of FSH. These relationships are summarized in Table 3.

DISCUSSION

The pituitary glands of male rats with intact gonads contain little or no LH; whereas those of female rats with intact gonads contain both FSH and LH. Removal of the gonadal influence by castration of either sex results in a marked increase in LH as well as FSH in the pituitary. Whether this is primarily the result of reduced liberation, or of increased production, is not known. It is well known that in women following ovariectomy, or in the menopause when ovarian function is diminished, the gonadotropic activity in the blood and urine is predominantly FSH. This has also been found true for rats following castration (8). It would thus appear that the liberation of LH from the pituitary is markedly decreased in the absence of gonadal secretions.

If, however, gonadectomized rats are injected with sufficient estrogen for a period of 45 days, the pituitary LH which had increased following castration is, in most instances, no longer demonstrable. Thus estrogen apparently induces the liberation of LH. The concentration of LH has been found to increase in the urine of menopausal women during the early stages o

estrogen treatment (16). In the same women, FSH decreases in the blood and urine under estrogen influence. The changes in concentration of these substances in the pituitary, as well as in the blood and urine, may well depend upon the time when the observations are made.

The effects of androgens are similar to those of estrogen in some respects, yet different in others. Androgens, like estrogens, induce release of LH from the pituitary, and decrease the concentration of FSH in the blood and urine. But under androgen influence the residual pituitary FSH remains high. Greep and Jones (10) have reported that pituitary FSH increased in intact female rats following testosterone treatment. Using a different experimental method, Hoogstra and Paesi (17) stated that the administration of two mg. of testosterone daily increased the pituitary FSH content of intact and gonadectomized rats of both sexes. However, recently Paesi *et al.* (18) reported that if the daily dose of testosterone propionate was limited to 100 μg., the pituitary FSH content of the intact female was increased but not that of the intact male or the gonadectomized animal of either sex. It thus appears that androgen may not induce release of FSH from the pituitary, whereas estrogen interferes with FSH production as well as its release. Therefore in considering the reciprocal relationships of gonadal and pituitary hormones, it is essential to consider the *production* of each pituitary factor independently of its *release;* the commonly used descriptive terms "inhibit" and "suppress" are not adequate.

In this investigation, all of the pituitary glands from rats that had been gonadectomized but received no gonadal hormone treatment, induced luteinization in the ovaries when injected into immature animals. With a few exceptions, the pituitary glands of the castrated donor rats receiving the equivalent of 3 μg. of estradiol benzoate or 3 mg. of testosterone propionate per day for 45 days, induced no visible luteinization in the ovaries of most of the test rats. The question thus arises: why did the pituitaries from a few of these latter animals, or from others receiving higher dosages of estrogen or androgen, continue to induce luteinization? Regardless of the level to which the dosage of estrogen or androgen was raised, the point was never reached where the ovaries of *all* of the test rats were completely free of lutein tissue. This could be explained in part by individual variation in sensitivity of the ovaries of the test animals to pituitary stimulation, or by interference by the test animal's own pituitary in the normal animals or in those where hypophysectomy might through inadvertence not have been complete.

Individual variation in the responsiveness of the donor rat's pituitary to the influence of the gonadal steroids must also be considered. Such a variability exists in women, who do not all exhibit consistently high urinary FSH titers following bilateral ovariectomy.

It has been held that the chemical fractionation of gonadotropins into separate FSH and LH factors is an artifact resulting from chemical treat-

ment. But the differences in ovarian responses observed in this study could not be due to chemical manipulation, since the treatment was uniform and was limited to desiccation by use of acetone. In another study, not reported, some of the pituitaries were merely macerated between two glass slides and permitted to airdry instead of being desiccated. The results induced by these air dried pituitaries were similar to those of pituitaries desiccated in acetone.

Since an unpublished study assaying FSH preparations by both ovarian follicle development and stimulation of mice uteri showed that there was no correlation between results of these two procedures, the criteria used in the present study for evaluating changes in the pituitary hormones have been 1) for FSH, follicle development and 2) for LH, luteinization in the ovaries of the immature recipient rats. The differences in uterine and ovarian responses to gonadotropic stimulation may account in part for the disparity of published results regarding the measurement of pituitary FSH. Quantitative or qualitative evaluation of FSH by uterine response has some acceptance, but is open to question. FSH does not stimulate the uterus directly; and it is doubtful whether purified FSH alone will stimulate the uterus indirectly through the ovary, since estrogen production is lacking (19).

Functional activity of the ovary is influenced by the interaction of varying amounts of FSH and LH, together with a number of other "augmenting substances" (20, 21, 22). Just as ovarian response is modified by substances other than pituitary gonadotropins, changes in the uterus may likewise not be solely dependent upon ovarian secretions.

The effects of pituitary, blood, and urinary substances on the uterus in the intact animal represent a composite action both directly and indirectly through the ovary, of a number of stimulating or modifying factors, of which FSH is but one. The terms "follicle stimulating" and "luteinizing" can apply only to the gonad. No objection would of course be made to calling the effects on the mouse uterus, "uterotropic" or effects on ventral prostate of the rat "prostatropic," or to using such procedures to assay certain gonadal hormones. It would be desirable to evaluate *functional* changes in ovarian target organs, indirectly produced by pituitary hormones, but no satisfactory criteria exist at the present time for such measurements.

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NOTES AND COMMENTS

THE RESPONSE OF HYPOPHYSECTOMIZED MICE TO INJECTIONS OF HUMAN SERUM CONTAINING LONG-ACTING THYROID STIMULATOR

ABSTRACT

Human serum, obtained from a patient with Graves's disease and containing a high level of long-acting thyroid stimulator, elicited highly significant responses when injected into hypophysectomized mice. The mean response did not differ significantly from that elicited in a control group of intact mice. This shows that the site of action of the abnormal thyroid stimulator is not the pituitary gland and makes it likely that the material acts directly on the thyroid.

Serum from cases of Graves's disease has been shown to contain a thyroid-stimulating substance which differes from thyrotrophin in that it stimulates thyroid secretion for a longer time after a single injection (1, 2, 3). This long-acting thyroid stimulator has in the past been called the "abnormal thyroid-stimulating hormone" and the "abnormal thyroid stimulator" (4).

The experiment reported below shows that the presence of the pituitary gland is not necessary for the action of long-acting thyroid stimulator.

METHODS

Mice were injected with 1.5 μC of I^{131} and, 12 hours later, with 10 μg. L-thyroxine. Thereafter they were given drinking water containing a pancreatic digest of thyroid tablets, diluted to contain 130 mg. of thyroid substance (B.P.) per 100 ml. On the second and third days after the I^{131} injection, half the mice were hypophysectomized by a method based on that of Thomas (5). On the fourth day, 0.05 ml. of blood was taken from each mouse by cutting a tail vessel. The mice were then injected with 0.1 ml. of serum from a patient with Graves's disease (containing a high level of long-acting thyroid stimulator), or 0.1 ml. of serum from a euthyroid person, or 0.1 ml. of saline. Sixteen hours later the mice were bled a second time. The radioactivity of the blood samples was measured. The response to the material injected was the percentage change in the mouse's blood radioactivity.

At the end of the experiment the completeness of hypophysectomy was checked by making a post-mortem inspection of the sella turcica using a dissecting microscope. Results which had been obtained from animals which were found to have a visible pituitary remnant were discarded.

Using I^{131}, we have tested the thyroid activity of mice hypophysectomized in our laboratory, finding it to be greatly reduced. Three weeks after the operation, 14 mice which were subsequently found to have no visible pituitary remnants had a mean 24-hour thyroid uptake of $1.2 \pm 0.2\%$, compared with $18 \pm 3.7\%$ in 9 unoperated controls. An operated animal with a large pituitary remnant had an uptake of 22%.

RESULTS

Hypophysectomized mice do not tolerate intravenous injection of serum as well as do intact mice. Therefore, we made control injections of euthyroid human serum in addition to control injections of saline. The level of thyrotrophin in euthyroid human serum is too low to elicit significant responses in the assay animals (6), and none were obtained on this occasion (Table 1).

Long-acting thyroid stimulator elicited highly significant responses in both the hypophysectomized and the intact mice (Table 1). The mean response in the hypophysectomized mice was 481. That in the intact mice was 431. These values do not differ from each other significantly.

Received May 16, 1960.

TABLE 1. THE 16-HOUR RESPONSES[1] ELICITED BY SERUM FROM A PATIENT WITH GRAVES'S DISEASE (CONTAINING A HIGH LEVEL OF LONG-ACTING THYROID STIMULATOR) AND BY EUTHYROID HUMAN SERUM WHEN INJECTED INTO HYPOPHYSECTOMIZED AND INTACT MICE

Material injected	Hypophysectomized mice		Intact mice		
	long-acting thyroid stimulator	Euthyroid serum	long-acting thyroid stimulator	Euthyroid serum	Saline
No. of mice	10	11	12	8	9
Mean response	481	−17	431	2	−12
P for difference	<0.001		<0.01		

[1] The response is the percentage change in the blood I^{131} level of the assay mice following injection of the material being assayed.

DISCUSSION

It is apparent that long-acting thyroid stimulator has undiminished activity in hypophysectomized mice. This shows that it does not act on the thyroid indirectly by stimulating pituitary thyrotrophin secretion. Therefore, it is probable that its site of action is the thyroid gland itself.

Out findings confirm the previous work of Munro (3), who found long-acting thyroid stimulator to be effective in three hypophysectomized mice.

Acknowledgments

We are indebted to Miss J. Hunter and to Mr. W. S. Cague for technical assistance.

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ADRENAL CORTEX FUNCTIONAL ZONATION IN THE WHALE (*PHYSETER CATADON*)[1]

ABSTRACT

Adrenal glands from adult sperm whales (*Physeter catadon*) were grossly separated into the three cortical zones. Each of these specimens was subjected to solvent extractions, column and paper chromatography. Chemical determinations of total recovered aldosterone, 17-hydroxycorticoids and 17-ketosteroids were made. Aldosterone was present in both the subcapsular and midcortical tissue in small quantities. 17-Hydroxycorticoids were recovered in all parts of the cortex, the greatest amount in the midcortical tissue. 17-Ketosteroids were recovered in all zones but the majority was present in the inner cortical tissue. These findings support the functional zonation theory in the largest of animals. The presence of aldosterone in the outer zones of the adrenal cortex even in mammals drinking sea water, is noted.

Evidence indicates that each of the zones of the adrenal cortex is concerned with secretion of different types of adrenal cortical hormones. The zona glomerulosa is primarily responsible for the secretion of aldosterone, the zona fasciculata for 17-hydroxycorticoids and the zona reticularis for 17-ketosteroids (1–13). Previous studies have been done on adrenals of the rat, the dog, man, beef and other species. Because of its size, the whale adrenal afforded the theoretical opportunity to separate the gland into the three zones for chemical analysis of each zone.

MATERIAL AND METHODS

Adrenal glands of adult sperm whales (*Physeter catadon*) were secured at Paita, Peru, South America.[2] One animal weighed approximately 21,708 kg. and measured 13.4 m. in length. Additional glands were obtained from other animals of comparable size. The whales were killed off the coast of Peru at a latitude South 56° and 15′ and longitude West approximately 81° and 32′. Ambient water temperature was about 21.1° C. The killed animals were towed into a shore-based factory and dissected within 18 hours after death. Adrenal glands were preserved by freezing and later, during transport, by immersion in 95% ethanol. The adrenal glands ranged from 180 to 272 gm. in weight.

Histologic sections were made. The remainder of each of 6 glands was separated by means of a razor blade into 3 zones consisting of the outer 4 mm. of the cortex including the capsular tissue, the midcortex including predominantly zona fasciculata and the inner cortex consisting predominantly of the zona reticularis. The separation into three zones was not perfect, there being undoubtedly some overlapping tissue present. Grossly recognizable medullary tissue was discarded.

The wet tissue was homogenized and extracted with acetone and then evaporated *in vacuo* to remove acetone. Methanol was added to the aqueous phase to a final concentration of 70%. This was partitioned with Skelly Solve B and the latter, containing neutral fats and lipids, was discarded. The aqueous methanol phase was then evaporated *in vacuo* and the remaining aqueous solution was extracted with chloroform. The chloroform residue was saved and subjected to silica gel column chromatography. The elutrients used for this purpose were mixtures of acetone and chloroform (1:99 and then 1:1) and finally acetone and methanol (1:1). Each solvent mixture was collected as a single fraction and dried *in vacuo*. These residues were subjected to 17-ketosteroid (Zimmerman chromogen) determination (14, 15); 17-hydroxycorticoid determination by the method of Silber-Porter (16) and paper chromatographic analysis utilizing both the Zafforoni (17, 18) and Bush (19) methods. Aldosterone isolated from paper chromatography, using compound M[3] (F) and Fa[3] (E) as markers, was cut out and eluted with methanol (20) and its concentration was estimated by Nowaczynski's blue tetrazolium color reaction (21).

RESULTS

Histologic study of the adrenal cortex showed considerable autolysis of the cells. The demarcation between zona glomerulosa and zona fasciculata was not a sharp line

Received June 15, 1960.

[1] Supported by Grants Nos. H-5069 and H-2336, National Institutes of Health, U. S. Public Health Service, Department of Health, Education and Welfare.

[2] Cia Ballenera del Norte, an affiliate of Archer-Daniels-Midland Company, Minneapolis, Minn.

[3] Following Reichstein's Lettering System for adrenocorticoids (Steroids, Fieser and Fieser, Reinhold Publishing Corporation, New York).

as in the rat, dog or beef, but was quite similar to the demarcation of the human adrenal gland. The results of the chemical analysis for aldosterone, 17-hydroxycorticoids and 17-ketosteroids are tabulated in Table 1. The amount of hormone recovered is reported as one figure rather than per 100 gm. of wet tissue because of the probability that some diffusion of hormones out of the glands into the ethanol occurred during their shipment. The percentage of total hormone recovered in separate zones showed that in each zone the majority of the hormone was of the 17-ketosteroid type. The percentage of total aldosterone recovered was slightly greater in the zona fasciculata than in the zona glomerulosa. The percentage of total 17-hydroxycorticoids recovered was greatest in the zona fasciculata. The percentage of 17-ketosteroids recovered was greatest in the zona reticularis.

TABLE 1. CHEMICAL DETERMINATION

Specimen origin And chemical determination	Total recovered hormones from 6 glands (μg.)	% of total hormones recovered in each zone	% of total aldosterone recovered from each zone	% of total 17-hydroxycorticoids recovered from each zone	% of total 17-ketosteroids recovered from each zone
Glomerulosa (Subcapsular tissue)					
Aldosterone	135.6	6.3	40.8		
17-Hydroxycorticoids	234.0	10.7		38.2	
17-Ketosteroids	1808.0	83.0			20.3
(Zimmermann chromogens)					
Fasciculata (Midcortical tissue)					
Aldosterone	196.4	7.5	59.2		
17-Hydroxycorticoids	261.0	10.0		42.6	
17-Ketosteroids	2162.0	82.5			24.3
(Zimmermann chromogens)					
Reticularis (Inner cortical tissue)					
Aldosterone	0	0	0		
17-Hydroxycorticoids	117.6	2.3		19.2	
17-Ketosteroids	4905.0	97.7			55.4
(Zimmermann chromogens)					

DISCUSSION AND INTERPRETATION

The results shown in Table 1 have several significant points. The whale represents a mammal which is adapted to drink sea water with its heavy load of sodium, chloride and other ions. Nevertheless aldosterone was present in significant quantities in both the subcapsular and midcortical tissue of the adrenal gland. The following explanation is postulated for a slightly greater percentage being recovered from midcortical tissue than from the subcapsular tissue: Greater diffusion of hormone probably occurred out of the subcapsular tissue into the surrounding ethanol than from the midcortical tissue. Secondly, the total sample of outer cortex was of less mass than the midcortex. Thirdly, the separation between these two zones was imperfect. It should be noted that no aldosterone was recovered from the inner cortical tissue.

The 17-hydroxycorticoids were recovered in all parts of the cortex, the greatest amount in the zona fasciculata, which is in keeping with previous studies on mammals. Undoubtedly there was some diffusion between the zones from the time the animal was killed until the adrenal glands were dissected as well as during shipment while preserved in ethanol. The 17-ketosteroids (Zimmerman chromogen) are of interest because of the relatively large amounts recovered in comparison with the amount of 17-hydroxycorticoids in all zones. But the majority of 17-ketosteroids was present in the zona reticularis.

In conclusion, the thesis of functional zonation of the adrenal cortex, previously proven in multiple species of smaller mammals, appears to be also true for the largest mammal.

Acknowledgments

We wish to express our gratitude to Dr. Rolland C. Reynolds, Mrs. Rita Douglas and Mr. Virgil Doty for their contributions to this project and also to express our appre-

ciation for the use of whaling facilities of Cia Ballenera del Norte and the Archer-Daniels-Midland Company.

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A NEW COLORIMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF STEROIDS CONTAINING THE 17α-KETOL SIDE CHAIN[1]

ABSTRACT

A color reaction has been described between a modified Nessler's reagent and a number of steroids containing the α-ketol side chain at C-17. The sensitivity of the reaction permits the ready quantitative colorimetric determination of a reactive steroid in amounts ranging from 10 to 90 μg./5 ml. solution. Nonreactive steroids did not interfere with color developed in the reaction of the reagent with a 17-ketol side chain-containing steroid. Application of the method to urine, and to the recovery of steroid from urine, is described.

A number of quantitative procedures have been developed for estimation of steroids containing the α-ketol grouping at C-17. These methods, based upon the reducing properties of the side chain, include the reduction of copper (1), ferricyanide (2, 3), phosphomolybdate (4) and tetrazolium salts (5).

The method described in this paper involves the reaction of the α-ketol side chain of steroids with a modified Nessler's reagent at pH 13.5, resulting in the production of a yellow color. The color intensity is directly proportional to the steroid concentration from 10 to 90 μg. of steroid. Steroids containing other functional groups but lacking the α-ketol side chain at C-17 do not react with the reagent under the conditions employed.

EXPERIMENTAL

Reagents

Nessler's reagent, prepared as described by Koch and McMeekin (6).
0.1N Acetic acid.
0.1% Aqueous methylcellulose (Dow Methocel).
Nessler-acetic acid reagent (prepared daily): 4 ml. Nessler's reagent, 2 ml. 0.1% methylcellulose, 16 ml. 0.1N acetic acid.
Ethyl ether: chloroform: 3:1 (v/v).
Ethanol purified as described by Sobel *et al.* (7).

Steroids used were highly purified reference samples purchased from the United States Pharmacopeia or from Dextran Chemicals, Inc. Steroids from other sources as indicated. Other chemicals used were analytical reagent grade.

Procedure

Determination of steroids containing the 17-ketol side chain.

To a solution of 10 to 80 μg. of steroid in 0.1 ml. ethanol is added 1.9 ml. H_2O followed by 3 ml. Nessler-acetic reagent. Mixing is performed by transferring the solution to another test tube or a cuvette. Within 15 seconds of mixing, the solution is placed in the dark and color allowed to develop for 8 to 15 minutes. The optical absorption of the yellow, turbid solution is determined at 425 mμ. against a blank containing all of the above reactants except steroid.

Application to urine.

Ten or 20 ml. urine are adjusted to pH 4.5 with dilute HCl and extracted twice with two volumes of ether-chloroform reagent. The volume of organic solvent is kept to a minimum by shaking the urine in 5 ml. portions with 10 ml. solvent. The extracted urine is pooled and shaken with a second 10 ml. of organic phase. Solvent phases are combined, extracted 4 times with 2 ml. 1N NaOH each time, and finally twice with 2 ml. portions of water. The organic solvent phase is transferred to a test tube and evaporated to dryness under a stream of warm nitrogen. The dried urine extract is then treated as described in preceding paragraph.

RESULTS AND DISCUSSION

Figure 1 shows that 10 to 90 μg. of cortisol can be measured conveniently in 5 ml. final solution.[2] Optical absorption of the yellow color formed with cortisol, shown in

Received June 15, 1960.

[1] This investigation was aided by grants from the National Science Foundation (G-5711) and the National Institutes of Health, United States Public Health Service (CY-3154).

[2] The following steroids which were studied at several concentrations also showed linear relationship between concentration and absorbancy: cortisone, corticosterone, Δ⁴-cortisone, Δ¹-cortisol, 9α-fluorohydrocortisone, 16α-hydroxy-9α-fluorohydrocortisone, 16α-hydroxy-9α-fluoro-Δ¹-hydrocortisone.

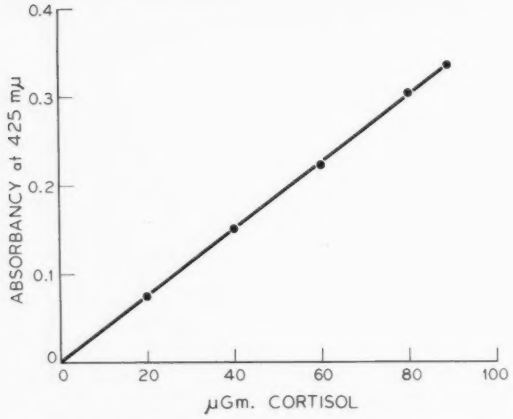

FIG. 1. Relationship between color intensity and cortisol concentration.

Figure 2, did not reach a maximum in the visible range. The course of color development with time is illustrated in Figure 3. In total darkness, maximum color intensity was achieved in 5 minutes. The absorbancy increased thereafter at the rate of 0.003 optical units per minute for the next five minutes. For this reason, to assure reproducible results, all tubes were read after the same time interval. Intensity of color developed in diffuse light was lower than that developed in total darkness and much less stable. Reproducible color development could not be obtained when the reaction was allowed to proceed in the light.

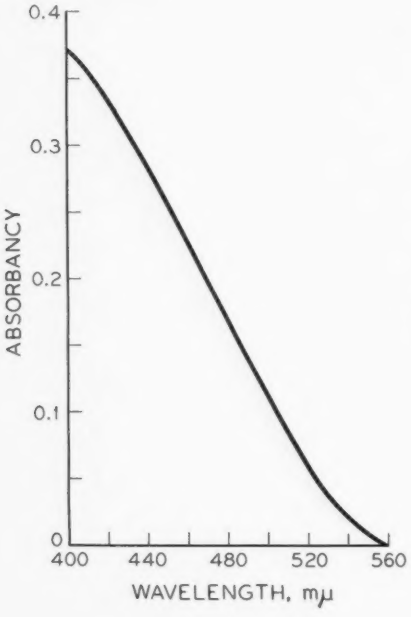

FIG. 2. Optical absorption of yellow color formed by reaction of cortisol with Nessler-acetic acid reagent.

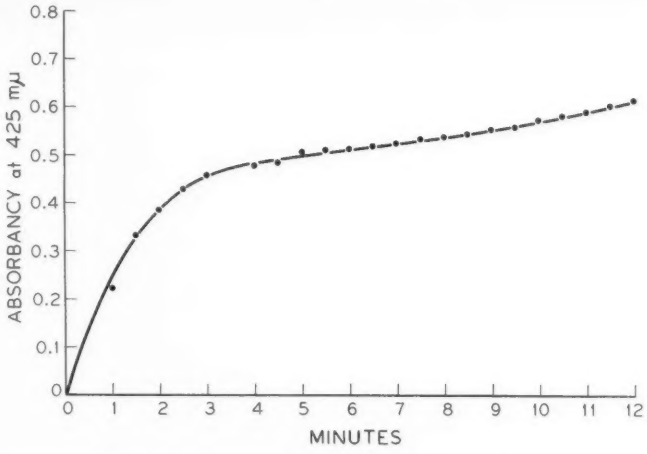

FIG. 3. Time course of development of yellow color formed by reaction of cortisol with Nessler-acetic acid reagent.

Studies of the reaction of steroids with Nessler's reagent were performed initially with higher concentrations of Nessler's reagent than described in the Procedure, and with either NaOH as H_2O as diluents. The color formed was variable, ranging from yellow to orange and with unpredictable intensity. It was found, however, that when the pH was adjusted to about 13.5 with acetic acid or hydrochloric acid, reproducible color intensities were obtained. Further reduction in pH sharply curtailed color formation.

In all experiments described above, 0.1% methylcellulose was added to the reagent to stabilize the suspension of HgI formed by the reaction of Nessler's reagent with steroid. When methylcellulose was omitted, the color formed during the reaction was markedly increased, almost doubling in intensity, but the degree of color was not reproducible. At the sacrifice of sensitivity, but with the gain of reproducibility, methylcellulose was used in all investigations.

Experiments were performed to determine which constituents of Nessler's reagent were responsible for the color reaction. Nessler's reagent is customarily prepared by mixing appropriate amounts of iodine, KI, mercury metal and NaOH to give an alkaline solution of potassium mercuric iodide. A mercury-free reagent was prepared by dissolving 2.25 gm. iodine in a solution of 3 gm. KI in water, diluting to 20 ml. with water and adding 48.8 ml. 10% NaOH. This solution gave no color wtih 100 μg. of cortisol. Furthermore, no color was produced with steroid and $Hg(NO_3)_2$, NaOH, or methylcellulose, alone or in combination. Therefore, the complete Nessler's reagent was essential for color formation.

Table 1 summarizes the results of a study of a number of steroids treated with the Nessler-acetic reagent under the conditions described. Only steroids having the 17-ketol side chain yielded color with the reagent. The color intensities per μg. of most reactive steroids, including the major adrenal steroids, lie close to that of cortisol. Molar extinction coefficients of the acetate derivatives were slightly higher than for the free alcohol forms. It may be noted that Triamcinolone and other 16,17-dihydroxy steroids containing the ketol side chain react with the Nessler-acetic acid reagent. These results are particularly interesting in view of the fact that these steroids could not be measured using the Porter-Silber reagent (8). The lack of reactivity of functions other than the 17-ketol side chain can be seen from the list of nonreactive steroids in Table 2. A sample

TABLE 1. RELATIVE COLOR INTENSITIES OF REACTIVE STEROIDS

Steroid	Absorbancy of 40 μgm. steroid	Average deviation	Micromolar extinction coefficient
Cortisol	0.266 (7)*	0.011	2.41
Cortisol 21-acetate	0.264 (6)	0.009	2.67
Cortisone	0.283 (6)	0.013	2.55
Cortisone 21-acetate	0.272 (8)	0.021	2.73
Δ^1-Cortisone	0.283 (2)	0.002	2.53
Δ^1-Cortisol	0.270 (3)	0.002	2.44
17α-Hydroxydeoxycorticosterone	0.241 (2)	0.003	2.09
17α-Hydroxydeoxycorticosterone acetate	0.230 (4)	0.020	2.23
Deoxycorticosterone	0.322 (4)	0.017	2.66
Deoxycorticosterone acetate	0.330 (4)	0.010	3.07
Corticosterone	0.330 (4)	0.002	2.86
Aldosterone†	0.349 (1)	—	3.15
16α-Hydroxy-9α-fluorohydrocortisone‡	0.27 (4)	0.012	2.65
9α-Fluorohydrocortisone‡	0.22 (3)	0.013	2.08
16α-Hydroxy-9α-fluoro-Δ^1-hydrocortisone (Triamcinolone)‡	0.30 (4)	0.008	2.93

The following steroids obtained from various commercial sources reacted with the Nessler-acetic reagent. Absorbance of 40 μg. steroid is given in parenthesis: 16α-hydroxydeoxycorticosterone (0.468); 2α-hydroxycortisone (0.468); D-homocortisone acetate (0.293); 6β-hydroxycortisone (0.282); 6-ketocortisol-21-acetate (0.282); Δ^{16}-dehydrodeoxycorticosterone (0.279); 17α-hydroxy-4-dihydrodeoxycorticosterone (0.271); Δ^5-pregnene-3β-21-diol-20-one (0.240).

* Number of experiments in parentheses.
† Generously supplied by Dr. Sam Solomon.
‡ Generously supplied by Dr. Josef Fried.

of 6-ketoprogesterone obtained from Dr. Sam Solomon yielded a faint color with modified Nessler's reagent equivalent to 0.08 absorbancy units per 40 μg. steroid.

When two chromogenic steroids were mixed, the resulting optical density with Nessler-acetic reagent was equal to the sum of the values contributed by each steroid (Table 3). It is also evident from this table that nonreactive steroids did not interfere with the color intensity developed by reactive compounds. High concentrations of sugars did yield some color in the procedure. Four hundred micrograms each of fructose, glucose, mannose, and galactose gave, respectively, absorbancy values of 0.29, 0.13, 0.05, and 0.25. Glucuronic and citraconic acids gave no color with the reagent.

Recovery of steroids from water and urine was also studied. Using methylene chloride as extraction reagent, recovery of cortisol from water ranged from 83 to 122% with a mean of 98%. Recovery of added cortisol from urine was studied using as extraction

TABLE 2. STEROIDS NOT REACTING WITH MODIFIED NESSLER'S REAGENT

Allopregnente-3β-ol-20-one (S)*	Estradiol (S)
Androstane-3β-ol-17-one (S)	17-Methylene-Δ^4-androstene-3-one-17α-20-epoxide (S)
Δ^1-Androstene-3,17-dione (S)	19-Nortestosterone
Δ^4-Androstene-3,17-dione (S)	17α-methyl-19-nortestosterone
Androstane-3,11,17-trione (S)	Pregnanediol (Sch)
Δ^4-Androstene-3α,17β-diol (S)	Progesterone (Me)
Dihydrotestosterone (S)	Δ^4-Pregnene-6β-ol-3,20-dione (S)
21-Deoxycortisol (S)	Δ^4-Pregnene-3,11,20-trione-17α-ol-21-thiol acetate (S
16-Dehydroprogesterone (S)	21-Methoxypregnenolone (S)
16,17-Dihydroxyprogesterone (S)	Testosterone (S)
21,21-Dimethylprogesterone (S)	Testosterone ethyl ester (CS)
	Testosterone citraconate (S)

* Abbreviations in parentheses indicate source as follows: (S) Syntex, S. A.; (Sch) Schering Co.; (Me) Merck-Sharp & Dohme Co., (CS) Chemical Specialties, Inc.

TABLE 3. COLOR DEVELOPMENT WITH STEROID MIXTURES

A	Absorbancy at 425 mμ	*C*	Absorbancy at 425 mμ
1. Dihydrocortisone (0.28 μm.)	0.70	1. Testosterone (0.20 μm.)	0.07
2. Deoxycorticosterone (0.29 μm.)	0.78	2. Cortisone (0.13 μm.)	0.34
3. 1+2	1.50	3. 1+2	0.48
B		*D*	
1. Progesterone (0.50 μm.)	0.07	1. 6 β-Hydroxycortisone (0.26 μm.)	0.27
2. Cortisol (0.19 μm.)	0.45	2. Δ^1-Androstene 3,17-dione (0.20 μm.)	0.02
3. 1+2	0.56	3. 1+2	0.28

TABLE 4. COMPARISON OF NESSLER-ACETIC AND BLUE TETRAZOLIUM PROCEDURES FOR URINARY STEROIDS

	Reagent	ml. urine	No. of expts.	μg. steroid found
Expt. 1	Nessler-acetic acid	10	3	15 ±1.0*
	Blue tetrazolium	10	3	11 ±1.5
Expt. 2	Nessler-acetic acid	20	3	23.5±1.6
	Blue tetrazolium	20	3	23 ±0.5

* Means and average deviations.

solvents either chloroform, methylene chloride, or a mixture of ethyl ether and chloroform 3:1. The last named solvent mixture gave the best results, it being the only one which consistently did not form emulsions with urine. Recoveries of cortisol from urine using the ether-chloroform solvent were 89 and 98 percent in two experiments.

Estimation of steroids in urine was made using the procedure described in this paper and the blue tetrazolium method (5). The data in Table 4 indicate that similar data were obtained by the two methods applied to the same samples.

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LACK OF A DIRECT ADRENOTROPIC ACTION OF CHORIONIC GONADOTROPIN ON THE RAT ADRENAL[1]

ABSTRACT

There is conflicting evidence on a possible adrenotropic action of chorionic gonadotropin (CG). We have recently found an adrenotropic action of CG in hypophysectomized male rats, but this action was mediated by the testes. We have now attempted to find whether CG had, in addition, a direct effect on the rat arenal; the data gave no indication of such an action. We believe, therefore, that the adrenotropic action of CG is confined to that mediated by the testes.

Several investigators (1, 2, 3) have believed that gonadotropins were adrenotropic and in particular that they stimulated adrenal androgen production. Experimental work (4) has on the whole failed to support this concept, but our interest in this question was roused by indirect evidence (5) that chorionic gonadotropin (CG) might be related to the enlargement of the adrenal which occurs normally in the fetuses of certain primates, including man. We later found (6) that CG had limited adrenotropic properties in hypophysectomized rats; this action proved to be an indirect effect mediated by the testes and did not appear to be related to a possible effect in primates. We wished therefore to determine whether CG had in addition a direct influence on the rat adrenal. We have tested it in high doses in castrate rats, both hypophysectomized and non-hypophysectomized, and have found no evidence of a direct action on the adrenal gland.

METHODS

Cesarian derived rats of Charles River strain castrated or castrated and hypophysectomized on the 30th day of age were obtained from Charles River Laboratories. The animals were individually caged and selected by random number for treatment groups; there were at least 6 rats in each group. Injections were started the day after operation and continued for 14 days; all injection material was given subcutaneously in 0.3 ml. volume. Testosterone propionate was given once daily in sesame oil; CG was given three times daily in human plasma. Rats were killed with ether on the morning following the last injection. Organs were dissected, trimmed, and weighed. Adrenotropic activity was evaluated by adrenal weights, and androgen production by seminal vesicle and ventral prostate weights.

RESULTS

Treatment with 1000 I.U./day of CG in castrate and hypophysectomized-castrate rats produced no significant changes from control values in adrenal, seminal vesicle, or ventral prostate weights (Table 1). In other animals, testosterone propionate produced increases in seminal vesicle and ventral prostate weights; the minimum effective dose was between 5 μg. and 25 μg./day (Table 1).

DISCUSSION

CG in dose of 1000 I.U./rat/day was incapable of influencing the weights of the adrenals or the male accessory sex organs in castrate rats, either hypophysectomized or nonhypophysectomized. These findings failed to reveal a direct adrenotropic action of CG in either the involuting gland or in the gland under physiologic ACTH stimulation. Thus there was no evidence that steroid biogenesis was either stimulated or significantly altered toward a greater relative production of adrenal androgens. Adrenal androgen production during CG administration was biologically equivalent to less than the activity of 25 μg./day of administered testosterone.

These findings contrast with the demonstrated ability (6) of CG in doses of as little as 1 I.U./rat/day to partially maintain adrenal weights in freshly hypophysectomized male rats with intact testes. This response could not be elicited in castrate animals, but

Received June 22, 1960.

[1] These studies were supported by grant (A-1437) from the National Institutes of Health, Public Health Service.

TABLE 1. THE EFFECT OF CHORIONIC GONADOTROPIN AND TESTOSTERONE PROPIONATE ON THE ADRENALS, VENTRAL PROSTATE, AND SEMINAL VESICLES OF CASTRATE AND CASTRATE-HYPOPHYSECTOMIZED MALE RATS

Animals	Treatment	Daily dose	Adrenals	Organ weights (mg.)	
				Ventral prostate	Seminal vesicles
Experiment A					
Castrate	Plasma	0.9 cc.	47.2	11.8	9.6
Castrate	CG in plasma	1000 I.U.	48.6	11.0	9.2
Castrate, hypox	Plasma	0.9 cc.	13.8	7.5	7.6
Castrate, hypox	CG in plasma	1000 I.U.	11.5	8.5	7.2
Experiment B					
Castrate, hypox	Sesame oil	0.3 cc.	9.5	7.0	8.4
Castrate, hypox	TP in oil	5 μg.	9.3	10.6	11.5
Castrate, hypox	TP in oil	25 μg.	8.5	39.7	71.2
Castrate, hypox	TP in oil	100 μg.	10.6	120.2	322.0

Expt. A: CG failed to influence ($P > 0.05$) the organ weights in these animals.
Expt. B: Testosterone propionate in doses up to 100 μg. failed to influence adrenal weight ($P > 0.05$) but in dose of 25 μg. or 100 μg. caused an increase ($P > 0.01$) in ventral prostate and seminal vesicle weights. The weight increases with the 5 μg. dose were not significant ($P > 0.05$). These results indicate that CG in high dosage (1000 I.U./day) failed to stimulate the adrenal to produce androgen equivalent to 25 μg. of injected testosterone propionate.

could be reproduced in these animals by administration of large doses of testosterone and certain other steroids (7), most of which were androgenic. The minimal effective adrenotropic dose of testosterone propionate was about 500 μg/rat/day. Since these experiments show that adrenal androgen production during CG administration was equivalent to <25 μg/day of administered testosterone, it is clear that the adrenal was very far from producing sufficient androgen to influence its own weight. Our observations have led us to believe that the adrenotropic action of CG in the rat is confined to that mediated by the testes.

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Acknowledgments

The authors wish to acknowledge with thanks the help of Mrs. Lee Herrera of the Department of Medical Statistics, New York University, with evaluation of these results and the generous donation of chorionic gonadotropin by Dr. A. Stanley Cook of Ayerst Laboratories.

EFFECTS OF EXTRACTS OF HYPOTHALAMIC NUCLEI AND CEREBRAL CORTEX ON ACTH STIMULATING ACTIVITY IN THE DOG[1]

ABSTRACT

The effects of intracarotid injections of saline extracts of samples of certain hypothalamic nuclei or regions and motor cerebral cortex on blood levels of 17-hydroxycorticosteroid have been studied in the trained nonstressed dog. Extracts of 1 mg. of supraoptic nucleus and median eminence produced significant increases in 17-hydroxycorticosteroids while extracts of the same amounts of lateral hypothalamic nucleus, mammilary body, optic tract, paraventricular nucleus, and posterior sigmoid cerebral cortex failed to produce a significant increase.

Rumsfeld and Porter (1) and Royce and Sayers (2) recently reported that extracts of the hypothalamus possess ACTH releasing activity in the rat. This work confirms and extends the general findings of Slusher and Roberts (3) and De Wied *et al.* (4) who reported ACTH release following the administration of extracts of hypothalamic tissues.

Guillemin *et al.*[2] injected "purified" fraction from hypothalamic extracts from beef and hog hypothalamic tissues in dogs and humans and noted an increase in circulating as well as urinary 17-hydroxycorticosteroids.

In the investigation of Rumsfeld and Porter (1) the extracts were prepared from hypothalamic tissues extending from the anterior margin of the optic chiasm to the posterior margin of the mammilary bodies, 1 cm. to each side of the pituitary stalk. The mass of tissue employed by these workers obviously must include a great many hypothalamic areas for specific nuclei or different functional capabilities. The question quite naturally arises in all of these studies as to which of these areas or nuclei are actually concerned with the production of an ACTH stimulating substance.

In an effort to obtain more specific information on this problem, we have assayed the ACTH stimulating properties of tissues from several specific hypothalamic foci or nuclei in the dog.

MATERIALS AND METHODS

Hypothalamic tissues were removed from brains of 18 dogs according to the following procedures: The animals were anesthetized with sodium pentobarbital (35 mg./kg., I.V.) and placed on artificial respiration. The skull was opened and the calvaria removed. The brain was then quickly removed from the cranial cavity and a transverse section through the hypothalamus at the level of the middle of the optic chiasma was made with a single edged razor blade, thus dividing the brain into two portions and rendering the anterior hypothalamic nuclei on the caudal surface of the tissue accessible to removal by trochar. The pituitary gland was removed by transection at the level of the lower border of the pars tuberalis and the two portions of the brain were then plunged into liquid nitrogen. In some cases the freezing step was omitted.

Received June 28, 1960.

[1] Supported in part by research grant A-764 from the National Institute of Arthritis and Metabolic Diseases, in part by research grant B-146 from the National Institute of Neurological Diseases and Blindness, and in part by the University of Utah Research Fund.

[2] Guillemin, R., Hearn, W. and Clayton, G.: XXth International Physiological Congress—Communications, p. 378, 1956.

The tissues for testing were obtained by cylindrical "punch outs" from both frozen and unfrozen hypothalami. The punch outs were made with 13 to 15 gauge syringe needles ground by means of tapered silicon carbide abrasive points to form trochars with an inside bevel. Tissues from the following areas or nuclei were obtained for study: nucleus supraopticus, nucleus lateralis, nucleus paraventricularis, anterior median eminence, optic tract, mammillary body, and cerebral cortex (posterior sigmoid gyrus). The average wet weight of each punch out was about 12 mg. and a sufficient number of punches was made to provide about 50 mg. of tissue for each sample.

The tissues were allowed to thaw at room temperature at normal refrigeration temperature and then ground in a glass homogenizer with sterile saline, 1 ml. saline per mg. of tissue. After complete homogenation, the mixture was centrifuged at 3500 RPM for 30 minutes in the cold. The samples were then decanted and supernatant fluid used in the experiment. The supernatant fluid was assayed for the concentration of proteins by the method of Bucher (5) and from 50–100 μg. protein per mg. of brain tissue were found. All glassware used for homogenation and preparation of the tissues were sterilized before use.

Supernatant fluid from *1 mg. tissue* was diluted to 5 ml. with 0.85% NaCl and injected into the exteriorized carotid artery of trained dogs and assayed according to the method of Eik-Nes and Brizzee (6). The number of samples of extract from the various nuclei or areas varied from four in the case of paraventricular nucleus to 15 in supraoptic nucleus. ACTH stimulating activity was assayed in terms of plasma levels of 17-OHCS as described by Eik-Nes (7). All concentrations of 17-OHCS are expressed in μg./100 ml. plasma.

RESULTS

Mean peripheral blood levels of 17-hydroxycorticosterone increased significantly ($P<.01$) following injection of extract of nucleus supraopticus from a control of $1.5 \pm .07$ (standard error of the mean)/100 ml. to $3.7 \pm .15$/100 ml. in 15 minutes. Following injection of anterior median eminence extract the mean level again rose significantly ($P<.05$) from a control level of $1.4 \pm .07$ to $4.1 \pm .12$ in 15 minutes. In the remaining structures, the differences in control and the levels observed 15 minutes after the injection of test material were not statistically significant. The mean values obtained in each of these structures is as follows: lateral nucleus, $1.8 \pm .18$ control to $2.6 \pm .43$ following administration of extracts; optic tract, $1.7 \pm .20$ control to $2.1 \pm .20$ following administration of extract; paraventricular nucleus, $1.5 \pm .02$ control to $1.9 \pm .33$ following administration of extract; mammilary body, $2.1 \pm .24$ control to $2.6 \pm .31$ following administration of extract; and motor cortex, $1.6 \pm .11$ control to $2.1 \pm .25$ following administration of extract.

In order to compare results of frozen tissue extracts of supraoptic nucleus with extracts of fresh unfrozen tissues, a group comparison test ("t" test) was run on values obtained from each. The results ($P>.7$) indicated no significant difference between the results obtained from frozen or unfrozen tissues, the mean value being 3.8 μg./100 ml. plasma for the fresh tissues as compared with 3.73 μg./100 ml. plasma for the frozen tissue as noted above.

DISCUSSION

The results of this study support the findings of Rumsfeld and Porter (1), Royce and Sayers (2), and Hellerstein *et al.* (8) that hypothalamic extracts possess ACTH stimulating properties and suggest more specifically that the supraoptic nuclei and median eminence contain a higher concentration of such material than the other parts of the hypothalamus. Rather surprisingly, in view of the numerous reports indicating a neurosecretory function in the paraventricular nuclei, we found no indication of activity per 1 mg. sample of tissue for this area.[3] Further, we were unable to elicit any significant increase in plasma level of 17-hydroxycorticosterone from extracts of 1 mg. mammilary body, lateral hypothalamic nucleus, optic tract or motor cerebral cortex.

[3] This finding may support the observation of Fiske and Greep (9) showing cytological changes in the cells of the supraoptic nuclei in rats exposed to continuous light or darkness. Under these "stressful" conditions the cells of the paraventricular nuclei were unaffected.

Slusher and Roberts (3) on the other hand reported that a water-soluble non-protein substance present in anterior and posterior hypothalamus and cerebral cortex is capable of stimulating pituitary release of ACTH, using eosinopenia or adrenal ascorbic acid depletion as assay methods. A second factor capable of stimulating ACTH release which they conclude was a lipide or lipoprotein was present only in extracts of posterior hypothalamus. From the diagrams of the hypothalamic areas removed by these authors, it would appear that only a small portion of the supraoptic nucleus would have been included in the anterior hypothalamic segment since the block of tissue did not extend far from the midline. These findings could be at variance with our observations since saline extracts of 1 mg. cerebral cortex and mammilary body did not produce any significant increase in 17-hydroxycorticosterone levels following injection into the carotid artery in the trained dogs. However, it should be emphasized that our samples per injection were made from only 1 mg. of tissue whereas those of Slusher and Roberts were prepared from 25 gm.

The findings of De Wied *et al.* (4) appear to support those of Slusher and Roberts. However, De Wied and his associates reported that electrolytic lesions destroying the median eminence but not the hypophyseal stalk abolished the action of the lipide extract of posterior hypothalamus injected via the tail vein in the rat. This suggests that the principle elaborated by the posterior hypothalamus may require further conjugation in median eminence before acquiring the ACTH stimulating property.

The findings that compensatory adrenal hypertrophy does not occur in rats with lesions in anterior hypothalamus (10, 11) seems to be in agreement with our own results stressing the importance of anterior hypothalamic nuclei in the elaboration of the ACTH stimulating substance.

However, it has been clearly demonstrated that vasopressin will promote ACTH secretion in numerous animal species. Whether this is a direct effect on the pituitary gland (12), the adrenal gland (13) or both is still under debate. Since both the supraopticus (2) and the median eminence contain vasopressin-like material, the effect on 17-hydroxycorticosteroids seen in this investigation could actually be due to vasopressin. Furthermore, the median eminence is especially rich in histamine (14), and the occurrence of 3-hydroxytyramine in the dog's brain has been reported (15). Whether these substances are responsible for the "ACTH like" effect seen following the administration of extracts of small amounts of supraoptic or median eminence tissues seen in this investigation has not been tested.

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THE EFFECT OF HYPOTHALAMIC LESIONS ON ADRENAL SECRETION OF CORTISOL, CORTICOSTERONE, 11-DESOXYCORTISOL AND ALDOSTERONE[1]

ABSTRACT

The output of cortisol, corticosterone, 11-desoxycortisol and aldosterone was measured in the adrenal venous blood of normal dogs and dogs with hypothalamic lesions. Secretion of all these hormones was depressed in dogs with destruction of the median eminence, but unaffected by lesions elsewhere in the hypothalamus.

The original evidence that lesions of the median eminence of the hypothalamus depress the adrenocortical response to stressful stimuli included observations on circulating eosinophiles (1) and lymphocytes (2). Confirmatory reports have been based on measurements of adrenal ascorbic acid (3), circulating ACTH levels (4), and adrenal venous output of glucocorticoids (5) and Porter-Silber chromogens (6, 7, 8). The secretion of cortisol and aldosterone is depressed in animals with ventral hypothalamic lesions (9, 10), but the effects of brain lesions on other secretions of the adrenal cortex have not been described in detail.

MATERIALS AND METHODS

Stereotaxis lesions were produced in the median eminence in six male mongrel dogs. In another group of six dogs, other portions of the hypothalamus were destroyed, including the posterior tuberal region, the posterior hypothalamus, the ventromedial nuclei and the region around the anterior commisure. The lesions did not involve the midbrain or the pineal apparatus. Transbuccal hypophysectomy was performed in four additional dogs.

Six weeks after operation, blood was collected from the right lumbo-adrenal vein for two hours under ether anesthesia. The animals had been on a reduced sodium intake for 5 days. They were bled 20 ml./kg. from the femoral artery immediately before collecting the adrenal effluent, and the arterial blood was re-infused in amounts equal to the adrenal flow. Six similarly treated normal dogs served as controls.

Adrenal venous blood was collected in iced tubes, centrifuged, and the plasma promptly frozen. Subsequently, the plasma was extracted with chloroform. The extract was washed with 0.05N sodium hydroxide and water, then dried over sodium sulphate and evaporated under reduced pressure. The extract was chromatographed on paper in chloroform-formamide (11), and the areas corresponding to cortisol, aldosterone (or cortisone) and corticosterone eluted.

The 95% ethanol eluate of the aldosterone area from the chloroform-formamide paper was bioassayed in water-loaded, adrenalectomized rats. Details of the assays, the experimental methods, and the values for aldosterone secretion in these dogs have been reported elsewhere (10).

The cortisol fraction was rechromatographed in Bush's system C (12). The area corresponding to a cortisol standard was eluted with 95% ethanol. The Δ^4-3-keto-steroid was quantitated by absorption at 240 mμ. After treatment with concentrated sulphuric acid, this fraction showed an absorption spectrum characteristic of cortisol (11). The "corticosterone fraction" from the chloroform-formamide paper was rechromatographed in cyclohexane 100: dioxane 100: methanol 50: water 25 (13). The fractions corresponding to standards of corticosterone and 11-desoxycortisol (Reichstein's compounds S) were eluted in 95% ethanol. The Δ^4-3-keto-steroid concentration was measured by absorption at 240 mμ.

Received June 30, 1960.

[1] Supported by PHS research grants, A-630 and A-3062, from the National Institute of Arthritis and Metabolic Diseases, and a grant-in-aid from the Commonwealth Fund.

After treatment with concentrated sulphuric acid, these fractions showed appropriate ultraviolet absorption curves characteristic of authentic corticosterone or 11-desoxycortisol (11).

RESULTS AND DISCUSSION

Table 1 summarizes the steroid output values in normal dogs and dogs with hypothalamic lesions. Significant quantities of 11-desoxycortisol (Reichstein's compound S) were secreted by the dogs in each group. In the hypophysectomized dogs, aldosterone secretion averaged 0.6 μg./two hours (standard error 0.5 μg.). The levels of the other hormones were too low to measure accurately.

TABLE 1. ADRENOCORTICAL SECRETION IN NORMAL DOGS AND DOGS WITH HYPOTHALAMIC LESIONS. VALUES ARE MEANS ± STANDARD ERROR OF THE MEAN

Group	No. dogs	Hormone output (μg./2 hours)			
		Cortisol	Corticosterone	11-desoxy-cortisol	Aldosterone
Normal	6	297 ± 39	233 ± 55	67 ± 14	3.5 ± 1.0
Lesions sparing median eminence	6	308 ± 38	187 ± 41	64 ± 16	3.7 ± 0.5
Lesions destroying median eminence	6	158 ± 25	77 ± 15	28 ± 5	1.5 ± 0.4

Only those lesions which affected the median eminence depressed adrenocortical secretion appreciably. Outputs of all hormones were depressed to about the same extent. These findings are compatible with decreased action of ACTH on the adrenal, since ACTH probably acts on a step in steroid biogenesis prior to the differentiation of pathways leading to the several corticosteroids measured (14). None of these lesions showed an isolated effect on aldosterone secretion.

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THE EVALUATION OF DL-3′-IODOTHYRONINE AND DL-3′,5′-DIIODOTHYRONINE AS THYROXINE ANTAGONISTS IN THE RAT[1]

ABSTRACT

DL-3′-iodothyronine and DL-3′,5′-diiodothyronine were tested for their ability to inhibit the goiter-preventing effect of thyroxine in thiouracil-fed rats. They were shown to be without activity at a molar ratio of analog to L-thyroxine of 200 to 1.

DL-3′,5′-diiodothyronine was first prepared by Block and Powell (1) and tested for thyroxine-like activity by Salter.[2] The compound was found ineffective in elevating the oxygen consumption of a patient with myxedema at a dose 4 times an effective dose of thyroxine. The difficult synthesis precluded further study until Roche, Michel and Wolf (2) established conditions for the direct iodination of DL-thyronine to yield DL-3′-iodothyronine which could be further iodinated to DL-3′,5′-diiodothyronine. Both compounds were reported to be without thyroxine-like activity in the tadpole metamorphosis and rat antigoiter assays (2, 3).

The thyroxine-antagonistic effect shown by DL-2′,6′-diiodothyronine in the rat antigoiter assay (4, 5, 6) has focused attention on the potential antagonistic properties of thyronines lacking iodines in the 3- and 5-positions. With the reports (7, 8, 9) of antagonistic properties for DL-3,3′-diiodothyronine and DL-3,3′,5′-triiodothyronine, it was thought of interest to examine DL-3′-iodothyronine and DL-3′,5′-diiodothyronine for thyroxine antagonistic properties.

METHODS

The synthetic method of Roche, Michel and Wolf (2) was scaled to a larger size to provide amounts necessary for biological testing.

DL-3′-iodothyronine was prepared in 28% yield (816 mg.) from 2.0 gm. of DL-thyronine (Nutritional Biochemicals Corp.). It was essential that the original reaction mixture be concentrated to dryness *in vacuo*, bath temperature below 45°, with a stream of nitrogen passing through the solution. Evaporation in an air stream resulted in complete loss of iodinated product. The purification method of Roche was carried out twice to yield a product free of DL-thyronine. The compound dried over P_2O_5 (2 mm., 20°, 48 hr.), m.p. (uncor.) 201–203° (d.), lit. (2) m.p. 207° (d.). Analyses[3]: Calcd. for $C_{15}H_{14}INO_4$: C, 45.18; H, 3.51; I, 31.88. Found: C, 45.07; H, 3.72; I, 31.47. Ultraviolet spectra[4]: $\lambda_{max} = 292$ mμ ($\epsilon = 3260$) in 0.5N hydrochloric acid; $\lambda_{max} = 313$ mμ ($\epsilon = 4420$) in 0.5N sodium hydroxide. Chromatography: n-butanol: sat'd 2N ammonium hydroxide, Rf = 0.40, lit.(2) Rf = 0.41; n-butanol:acetic acid:water (78:5:17), Rf = 0.81, lit.(2) Rf = 0.68.

DL-3′,5′-diiodothyronine was prepared in 45% yield (238 mg.) from 400 mg. of DL-3′-

Received July 12, 1960.

[1] This investigation was supported by Research Funds of the University of California Academic Senate.

[2] Personal communication in Reference 1.

[3] Microanalytical Laboratory, Department of Chemistry, University of California at Berkeley.

[4] Ultraviolet spectra were prepared by Mr. Michael Hrenoff, Spectrographic Laboratory, University of California School of Pharmacy.

iodothyronine. The reaction mixture was concentrated as above under nitrogen. The purification method of Roche yielded a product, m.p. (uncor.) 194–197° (d.) which by elemental analysis and chromatography was shown to contain about 20% DL-3′-iodothyronine. DL-3′,5′-diiodothyronine was obtained in pure state by formation of the hydrochloride and precipitation of the free amino acid from aqueous solution with sodium acetate (1). The product dried over P_2O_5 (2 mm., 20°, 48 hr.), m.p. (uncor.) 197–200° (d.). Lit.(1) m.p. 206° (d.). Analyses[3]: Calcd. for $C_{15}H_{13}I_2NO_4 \cdot H_2O$: C, 33.17; H, 2.78; I, 46.73. Found: C, 33.17; H, 2.61; I, 44.80. Ultraviolet spectra[4]: $\lambda_{max} = 298$ mμ ($\epsilon = 3570$) in 0.5N hydrochloric acid; $\lambda_{max} = 321$ mμ ($\epsilon = 5880$) in 0.5N sodium hydroxide. Chromatography showed identity with an authentic sample[5]: n-butanol: sat'd 2N ammonium hydroxide, Rf = 0.53, lit.(2) Rf = 0.43; n-butanol :acetic acid:water (78:5:17), Rf = 0.83, lit.(2) Rf = 0.75.

The rat antigoiter assay was based upon the method of Cortell (5). Long-Evans male rats[6] were fed a standard powdered feed (Simsonsen Laboratories) containing 0.3% thiouracil. Following one day on this diet, daily subcutaneous injections of compounds in a volume of 0.25–0.40 ml. were carried out for 10 days. On the eleventh day the rats were sacrificed by chloroform inhalation, the thyroid glands cleaned under a dissecting microscope and weighed to the nearest 0.1 mg.

In the first assay compounds were dissolved in ethyl alcohol/propylene glycol (10/90, v/v) (10), plus one drop of concentrated hydrochloric acid when necessary, and stored between injections in the refrigerator at 3°. The compounds were found to be readily soluble in pure propylene glycol and this vehicle was used in the second bioassay.

RESULTS AND CONCLUSIONS

Table 1 shows that DL-3′-iodothyronine at a molar dose of 100 times an effective dose of L-thyroxine causes no significant reversal ($P > 0.80$) of the thiouracil-induced thyroid weight increase. This confirms earlier reports (2, 3) of a lack of thyroxine-like action for this compound. Both DL-3′-iodothyronine and DL-3′,5′-diiodothyronine caused no significant reversal ($P > 0.10$) of the antigoitrogenic effect at a molar dose 200 times that of concomitantly administered L-thyroxine. They therefore showed no thyroxine-antagonistic properties at this dose.

The absence of thyroxine-like or thyroxine-antagonistic activity for these compounds lacking substituents in the 3 or 3,5-positions of the alanine-bearing ring emphasizes the importance of this substitution for thyroid activity in the thyronine series. In this light it is tempting to speculate that the antagonistic 2′,6′-diiodothyronine interacts

TABLE 1. THE EFFECT OF DL-3′-IODOTHYRONINE AND DL-3′,5′-DIIODOTHYRONINE WITH L-THYROXINE ON THYROID WEIGHT OF THIOURACIL-TREATED RATS

Test No.	Compound injected*	Food	Avg. wt. of rats		Daily dose per 100 gm. of body weight		Molar ratio	Thyroid weight per 100 gm.	
			Initial	Final					
			gm.	gm.	μg.	μmole ×10⁹		mg.	±s.d.
1†	None	Untreated	102	135	—	—	—	8.5	0.8
	Vehicle	0.3% thiouracil	106	153	—	—	—	32.6	8.0
	Thyroxine	0.3% thiouracil	101	147	2.0	2.25	—	21.3	5.8
	Thyroxine	0.3% thiouracil	106	147	5.0	5.62	—	6.9	1.0
	Thyroxine plus DL-3′-iodothyronine	0.3% thiouracil	101	146	5.0 440.0	5.62 1124.0	— 200.0	7.0	1.3
	DL-3′-iodothyronine	0.3% thiouracil	120	162	220.0	562.0	100.0	28.1	4.3
2†	None	Untreated	185	235	—	—	—	13.7	5.5
	Vehicle	0.3% thiouracil	205	225	—	—	—	21.1	5.6
	Thyroxine	0.3% thiouracil	222	233	2.0	2.25	—	13.1	5.4
	Thyroxine	0.3% thiouracil	186	228	4.0	4.50	—	6.9	1.5
	Thyroxine plus DL-3′-iodothyronine	0.3% thiouracil	172	197	4.0 359.3	4.50 900.0	— 200.0	7.2	0.9
	Thyroxine plus DL-3′-5′-diiodothyronine	0.3% thiouracil	168	210	4.0 472.5	4.50 900.0	— 200.0	7.9	1.8

* Thyroxine, used as sodium L-thyroxine p ntahydrate, was provided by Dr. James Kerwin, Smith, Kline and French Laboratories.

† Five rats at each control and dose level.

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[5] A reference sample of DL-3′,5′-diiodothyronine was provided by Dr. Paul Block, J. We are grateful to Dr. Block for his valuable consultation in the purification of DL-3′,5′-diiodothyronine.

[6] From the Institute for Experimental Biology, University of California at Berkeley.

with a biological receptor site involved in the antigoitrogenic response so that the normal position of the rings is reversed. Thus the 2′,6′-iodines would become 3,5-iodines relative to the phenolic group which could then occupy the normal position of the alanyl chain in thyroxine.

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INHIBITION BY 6-METHYL-17-ACETOXY-PROGESTERONE OF ACTH SYNTHESIS AND RELEASE IN THE RAT[1]

ABSTRACT

Six-methyl-17-acetoxy-progesterone (SC9686) produced severe adrenal atrophy in rats following repeated injection. This atrophy was associated with depletion of pituitary ACTH concentration, implying reduction in ACTH synthesis. Inhibition of ACTH release in response to acute stress has also been observed following a single injection of SC9686. Acceleration of adrenal atrophy in hypophysectomized rats given SC9686 indicates an additional, extra-pituitary action of this steroid. Suppression of pituitary ACTH synthesis and release appear to be the predominant glucocorticoid effects of SC9686.

Six-methyl-17-acetoxy-progesterone (SC9686) is a synthetic steroid which, when administered orally or parenterally, exerts progestational effects in laboratory animals and in man (1, 2). Marked adrenal atrophy is also produced by this hormone (3, 4); this is a unique action for a progestational steroid and stimulated the following studies.

METHODS AND MATERIALS

Sherman rats, 100–125 gm., were maintained in a constant temperature animal room on Purina lab chow and water *ad libitum*. Hypophysectomized Sprague-Dawley rats, 65–70 gm., were obtained from commercial sources and injections were begun 24 hours post-hypophysectomy. Animals were sacrificed 24 hours following the last injection unless otherwise specified. Adrenal ascorbic acid was determined by the method of Mindlin and Butler (5). Methods for extraction and bioassay of pituitary ACTH have been previously described (6, 7). SC9686[2] was injected subcutaneously, 1 mg. per day, in 0.2 ml. peanut oil. Control animals were injected with oil alone.

Received July 13, 1960.
[1] This study supported by a grant from the National Institutes of Health A-2208(C).
[2] Supplied by Dr. C. Gantt, G. D. Searle & Co., Chicago, Illinois.

RESULTS

SC9686 produced adrenal atrophy in both male and female rats when injected for 7 and 14 days (Table 1). Body weight was diminished only in male animals. Pituitary atrophy was seen only in rats receiving SC9686 for 14 days. Ovarian atrophy was produced in 2 of the 3 female experimental groups receiving SC9686 alone. Thymus atrophy was not observed in any group injected with SC9686 alone. Rats receiving cortisone, 0.1 mg. daily for 7 days, did not manifest adrenal atrophy; this dosage of cortisone is equivalent to 1 mg. of SC9686 when liver glycogen deposition is used to assay glucocorticoid potency (4).

TABLE 1. EFFECT OF 6-METHYL-17-ACETOXY-PROGESTERONE (SC9686) ON ORGAN WEIGHTS IN RATS

	Treatment group	Body wt. (gm.)	Adrenal wt. (gm.)	Gonadal wt. (gm.)	Thymus wt. (gm.)	Pituitary wt. (mg.)
♀	SC9686 1 mg. ×7 d. (5)	133 ± 5‡	14 ± 1**	34 ± 3**	344 ± 42	4.2 ± 0 2
	Controls (oil) (5)	132 ± 4	26 ± 1	52 ± 4	376 ± 47	5.4 ± 0.5
♀	SC9686 1 mg. ×14 d. (10)	128 ± 4	15 ± 1**	37 ± 4**	—	3.9 ± 0.4**
	Controls (oil) (10)	121 ± 3	35 ± 2	56 ± 5		5.2 ± 0.3
♂	SC9686 1 mg. ×7 d. (8)	130 ± 4*	12 ± 1**	1001 ± 59	312 ± 30	4.1 ± 0.2
	Controls (oil) (6)	148 ± 6	27 ± 7	1270 ± 198	348 ± 40	4.3 ± 0.2
♀	SC9686 1 mg. ×7 d. (6)	126 ± 1	13 ± 1**	33 ± 4	352 ± 36	4.4 ± 0.1
	Cortisone 0.1 mg. ×7 d. (6)	124 ± 5	28 ± 2	49 ± 5	378 ± 42	5.3 ± 0.4*
	Controls (oil) (6)	120 ± 3	24 ± 2	30 ± 5	416 ± 19	4.0 ± 0.1
♀	ACTH, Zinc, 4 u ×7 d. (5) Oil 0.2 cc. ×7 d.	129 ± 2	41 ± 4**	49 ± 5	190 ± 36**	4.6 ± 0.9
	ACTH, Zinc, 4 u ×7 d. (5) SC9686 1 mg. ×7 d.	132 ± 2	43 ± 1**	44 ± 3	122 ± 10**	4.2 ± 0.4
	Controls (oil) (5)	132 ± 4	26 ± 1	52 ± 4	376 ± 47	5.4 ± 0.5

* $P < 0.05$ compared to control group.
** $P < 0.01$ compared to control group.
‡ Mean ± standard error of the mean.

Microscopic examination of adrenal glands from the SC9686 groups revealed atrophy involving all zones of the adrenal cortex, although the zona fasciculata showed the most striking changes. Severe lipid depletion was observed throughout the adrenal cortex with the exception of the zona glomerulosa.

The concurrent administration of SC9686 and ACTH produced the same degree of adrenal hypertrophy as did ACTH given alone (Table 1). The two ACTH-treated groups showed the expected atrophy of the thymus gland and also failed to develop the ovarian atrophy demonstrated by the group given SC9686 alone. The latter observation cannot be easily explained, although it may be analogous to the apparent trophic effect of cortisone on ovarian and pituitary size which occurred in the group of rats injected with 0.1 mg. of cortisone.

Pituitary ACTH concentration was significantly diminished in all groups receiving SC9686 (Table 2). No pituitary ACTH depletion was observed in the subgroup receiving 0.1 mg. of cortisone. Since the rats treated with SC9686 for 14 days developed pituitary atrophy, the depletion in pituitary ACTH content was greater than the depletion in ACTH concentration, the former being 55 per cent and the latter 73 per cent of control.

A single dose of 10 mg. of SC9686 in oil prevented the adrenal ascorbic acid depletion which was observed 4 hours after injection of 0.5 ml. of oil alone (Table 3, Exp't. A).

TABLE 2. DEPLETION OF PITUITARY ACTH CONCENTRATION FOLLOWING SC9686 TREATMENT

	Treatment group	Pituitary ACTH concentration*	Per cent of control value
♀	SC9686 1 mg. ×7 d. (5)	54 (42– 74)**	57%
	Controls (oil) (4)	95 (79–114)	
♀	SC9686 1 mg. ×14 d. (9)	85 (74– 99)**	73***
	Controls (oil) (9)	116 (102–135)	
♂	SC9686 1 mg. ×7 d. (8)	72 (60– 87)**	69
	Controls (oil) (5)	104 (89–123)	
♀	SC9686 1 mg. ×7 d. (5)	62 (48– 81)**	57
	Cortisone 0.1 mg. ×7 d. (5)	101 (83–141)	94
	Controls (oil) (5)	108 (78–132)	

* Milliunits per mg. wet pituitary tissue. Figures in parentheses are 95% confidence limits
** $P < 0.05$ compared to control group.
*** Pituitary ACTH content = 55% of control (see text).

another experiment, a group of animals was given the oil injection stress and half were unilaterally adrenalectomized 4 hours later. Ascorbic acid levels 6 hours following the oil injection alone were still depleted, whereas ascorbic acid depletion did not occur in the SC9686-treated animals between the fourth and sixth hours despite the added stress of unilateral adrenalectomy (Table 3, Exp't. B).

Administration of SC9686 for 1 week produced significant adrenal atrophy in a group of hypophysectomized female rats (Table 4). This atrophy remained significant following analysis of covariance with the body weights of the two groups which also differed significantly. Another group which had received oil injection but which were not sacrificed until 8 days after the last injection showed a more profound degree of adrenal atrophy, and also ovarian atrophy. As in the previous experiments, SC9686 treatment did not result in atrophy of the thymus gland. Completeness of hypophysectomy was assured by post-mortem craniotomies in several animals from each group.

TABLE 3. EFFECT OF SINGLE DOSE OF SC9686 ON ASCORBIC ACID DEPLETION FOLLOWING STRESS

Exp't A. 0.5 ml. oil injection used as stress; adrenal ascorbic acid determined 4 hours post-injection

Group	Adrenal ascorbic acid[1]
Uninjected controls (5)	501 ± 17
Oil injection alone (5)	383 ± 23[2]
Oil injection plus 10 mg. SC9686 (5)	454 ± 16

Exp't B. 0.5 ml. oil injection; left adrenalectomy 4 hours post-injection; right adrenals obtained 6 hours post-injection

Group	Adrenal ascorbic acid[1]		
	Left	Right	Δ
Oil injection alone (4)	330 ± 28	300 ± 40	−30 ± 40
Oil injection plus 10 mg. SC9686 (4)	435 ± 28[4]	523 ± 47[3]	+88 ± 27[3]

[1] Micrograms per 100 mg. adrenal weight. Mean ± standard error.
[2] Differs significantly at 5% level of confidence when compared to the 2 other experimental groups.
[3] Differs significantly at 1% level of confidence from oil injection group.
[4] Differs significantly at 5% level of confidence from oil injection group.

TABLE 4. EFFECT OF SC9686 ON ORGAN WEIGHTS IN HYPOPHYSECTOMIZED FEMALE RATS

Treatment group	Body wt. (gm.)	Adrenal wt. (mg.)	Ovarian wt. (mg.)	Thymus wt. (mg.)
Control Group 0.2 cc. oil × 7 days n = 8	72 ± 2	11.7 ± 0.4	14.1 ± 0.5	193 ± 22
SC9686 1 mg. × 7 days n = 9	68 ± 1*	9.8 ± 0.3**	13.4 ± 0.7	165 ± 27
2 week Controls 0.2 cc. oil × 7 days No Rx × 7 days n = 9	77 ± 2	8.9 ± 0.3**	7.9 ± 0.7**	182 ± 2

* $P < 0.05$ compared to control group.
** $P < 0.01$ compared to control group.

DISCUSSION

SC9686 appears to be a potent suppressor of both synthesis and release of ACTH by the anterior pituitary, and in this respect resembles glucocorticoids such as cortisone (7, 8). Although SC9686 exerts other glucocorticoid effects (anti-inflammatory assays, liver glycogen deposition), it is considerably less potent, as measured by these parameters, than hydrocortisone (3). The partial dissociation between pituitary-inhibition and other glucocorticoid actions which is exhibited by SC9686 apparently is not related to its progestational activity, since other potent progestational compounds such as 17α ethinyl, 19-nortestosterone do not cause adrenal atrophy. No certain correlations between structure and function can be inferred, although it is worth noting that neither the 11 or 21 position is hydroxylated in this steroid, in contrast to most other natural and synthetic glucocorticoids. Since this compound has been employed in clinical medicine for over 2 years without adrenal hypofunction yet being reported, the pituitary suppression seen in the rat may be species-related.

To the extent that the two studies can be compared directly, the data in Table 1 confirms that of Glenn *et al.* (3) concerning the failure of SC9686 to produce adrenal atrophy when ACTH is administered concurrently. However, the data obtained in hypophysectomized animals and summarized in Table 4 indicate that SC9686 may induce adrenal atrophy, in part at least, by an extra-pituitary effect, possibly through a direct action on the adrenal cortex. Although this action is probably less important quantitatively than the pituitary-suppression induced by SC9686, the conclusion of Glenn *et al.* that this steroid produces adrenal inhibition *only* in the presence of an intact pituitary gland is not supported by this experiment.

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MAINTENANCE OF PREGNANCY WITH PROLACTIN OR PROGESTERONE IN RATS ON A SUCROSE DIET[1]

ABSTRACT

The fecundity of female rats on a diet of sucrose and distilled water during pregnancy was compared to rats on the same diet receiving daily injections of 4–6 I.U. prolactin or 5 mg. progesterone. At autopsy on the 21st day after mating there was an average of 8.1, 10.8, and 10.0 implantation sites which included 2.2, 7.6, and 7.8 living young per rat in the control, progesterone, and prolactin groups, respectively. It is suggested that rats on a diet of only sucrose have a deficiency of prolactin.

It has been shown that exogenous progesterone maintains pregnancy in females on various deficient diets which alone will not sustain pregnancy. It was suggested that there were inadequate levels of endogenous progesterone when the animals were on deficient diets (7). This experiment was conducted to determine whether or not the luteal tissue of rats on a sucrose diet could respond to injected prolactin by secreting levels of progesterone high enough to maintain pregnancy.

METHODS

Young female rats of the Holtzman strain were randomly placed in one of three groups on the day of mating as determined by the presence of vaginal plugs. All groups were fed sucrose and distilled water ad libitum. One group received daily injections of 4–6 I.U. of prolactin,[2] one group received daily injections of 5 mg. progesterone, and the other received no treatment.

Body weights were recorded every other day. The females were autopsied on the 21st day after mating. The numbers of corpora lutea, living and dead young, and implantation sites were recorded.

RESULTS AND DISCUSSION

The results are shown in Table 1. The weight losses of the females for the 21 days after mating were 26, 22, and 25% for the prolactin, progesterone and no treatment groups, respectively. The fetus weights were 69% of those from females on a complete diet and similar to those on a low mineral diet (7).

It has been previously reported some rats were able to carry living young on a diet of sucrose alone and injections of progesterone greatly increased the number of females carrying young and the average number of young per female (7).

Received August 2, 1960.

[1] An abstract of this paper was presented at the Meetings of the American Society of Animal Production, 1956.

[2] The authors wish to thank Armour Laboratories, Inc., for providing the prolactin preparation.

TABLE 1. THE EFFECT OF PROGESTERONE AND PROLACTIN INJECTIONS ON THE MAINTENANCE OF PREGNANCY IN THE SUCROSE FED RAT

No. mated	Treatment	Wt. loss	No. preg. at time of autopsy.	Young per female			Avg. fetus wt. (gm.)
				Living	Dead	Sites	
13	None	25%	3	2.2	0	5.9	2.4
5	Progesterone	22%	5	7.6	2.8	0.4	2.9
5	Prolactin	26%	4	7.8	2.0	2.0	2.1

These results confirm the above findings and extend them to prolactin. The number of living young in the prolactin and progesterone injected groups were similar but somewhat below the average of 9.4 for this strain on a complete diet (7). The numbers of females having living young were 4 out of 5, 5 out of 5, and 3 out of 13 for the prolactin, progesterone and no treatment groups, respectively.

The sites listed in Table 1 are the implantation sites which have no embryonic or placental remnants. The average number of young living and dead was 10.4, 9.8, and 2.2 for the progesterone, prolactin and no treatment groups, respectively. This indicated that normal numbers of embryos implanted and were maintained for a longer time in the treated than in the untreated females.

These results suggest that the luteal tissue of the rat fed only sucrose during pregnancy was capable of secreting more progesterone under the luteotrophic action of prolactin. Also, it seems that the feeding of only sucrose caused a deficiency in the secretion of prolactin which caused a decrease in the progesterone secretion and the resultant loss of fetuses.

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THE EFFECT OF CORTISOL ON GLUCOSE UPTAKE BY RAT EPIDIDYMAL FAT PADS[1]

ABSTRACT

The *in vitro* rate of glucose uptake by epididymal fat pads removed from adrenalectomized rats is decreased if the rats have been previously injected with cortisol. Significant effects in 150–200 gm. rats are obtained with 0.5 mg. cortisol, given in a single subcutaneous injection. The effect can be observed within 1.5 hours after injection.

Recent *in vivo* experiments with fasted, adrenalectomized rats (1) have led to t

Received August 18, 1960.

[1] This investigation was supported by a research grant (A-3535) from the Nation l Institute of Arthritis and Metabolic Diseases, Public Health Service.

TABLE 1. RATE OF GLUCOSE UPTAKE BY EPIDIDYMAL FAT PADS IN VITRO AT VARIOUS TIMES AFTER ONE SUBCUTANEOUS INJECTION OF 2.5 MG. CORTISOL

At the stated times after injection the rats were killed, and the fat pads incubated as described in the text. The control group was injected 1.5 hours with suspension medium. Glucose uptake in μmoles/gm. wet weight/2.5 hours incubation. Each value is the mean from a group of five rats, with standard deviation of sample.

Time after injection (hours)	Glucose uptake
0.5	1.47 ±0.53
1.5	1.21 ±0.23
Control	1.83 ±0.54

tentative conclusion that one of the earliest effects of injected cortisol is to reduce the rate of glucose uptake by epididymal fat pads. In the experiments reported here it is now shown that the *in vitro* rate of glucose uptake by epididymal fat pads is reduced when the rats from which fat pads are removed have been injected with cortisol.

Male Sprague-Dawley rats (Charles River Breeding Laboratories) weighing 150–200 gm., were used on the fourth day after adrenalectomy. The results in Table 1 and in Table 2, Expt. I were obtained with rats that were fasted for about 24 hours before, and during, the experiments; those in Table 2, Expt. II, with rats that were not fasted. Expt. I and Expt. II were run at different times, with different groups of rats, so the absolute values obtained are not comparable. Subsequent to adrenalectomy the rats were given 0.9% sodium chloride to drink. Cortisol (California Corp. for Biochemical Research) was injected subcutaneously, in 1.0 ml. suspension medium (Upjohn No. 98 Sterile Vehicle). Control rats received 1.0 ml. suspension medium. Epididymal fat pads were removed and incubated in Krebs-Ringer bicarbonate medium, following in general the procedures described by Winegrad and Renold (2), but with certain variations of detail: both fat pads from each rat were incubated together in a 25 ml. Erlenmeyer flask, with 1.0 ml. medium containing initially 2.8 μmoles glucose (50 mg. per 100 ml.). Glucose uptake was determined by measuring the decrease in glucose concentration in the medium after 2.5 hours incubation. The concentration of glucose was determined by the glucose oxidase method ("Glucostat," Worthington Biochemical Corp.).

Two parameters of the cortisol effect have been studied so far: the rate of onset of the effect, and the dependence on the amount of cortisol injected. The data in Table 1 show that by 1.5 hours after injection of 2.5 mg. cortisol, the decrease in the rate of glucose uptake is clearly established (at a significance level of $P < .05$). When the rates of glucose uptake are measured 2.5 hours after injection (Table 2), highly significant decreases are

TABLE 2. RATE OF GLUCOSE UPTAKE BY EPIDIDYMAL FAT PADS 2.5 HOURS AFTER ONE SUBCUTANEOUS INJECTION OF VARIOUS AMOUNTS OF CORTISOL

Rats were killed 2.5 hours after injection, and the fat pads incubated as described in the text. Glucose uptake in *μmoles/gm. wet weight/2.5 hours incubation. Each value is the mean (five rats per group in Expt. I, six in Expt. II), with standard deviation of sample

Experiment	Cortisol injected (mg.)	Glucose uptake
I (fasted)	Control	2.81 ±0.30
	0.1	2.44 ±0.66
	0.5	2.06 ±0.38
	2.5	1.17 ±0.25
II (not fasted)	Control	1.66 ±0.10
	2.5	1.04 ±0.30

obtained with 0.5 and 2.5 mg. cortisol ($P<.01$ and .001 respectively, the latter value holding both for Expt. I and Expt. II). These conclusions remain essentially unaltered if the rates of glucose uptake are expressed in terms of the defatted dry weight of tissue (3), rather than in terms of wet weight.

It is not clear at present whether cortisol acts directly on the fat pad, either independently or perhaps in conjunction with other hormones, or whether it acts at some site remote from the epididymal fat pads. Perhaps of some significance in this respect is the report of Correa *et al.* (4) that the *in vitro* stimulation of glucose uptake produced by insulin in fat pads is reduced by prior injection of the donor rat with cortisone, no effect being observed unless insulin was added; however, their experimental conditions were sufficiently different from those employed here that a strict comparison of results is not possible at this stage.

A more extensive report of these findings will be presented in a subsequent publication.

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BLOOD AS A SITE FOR THE CONVERSION OF THE STEROID HORMONES[1]

ABSTRACT

Blood from bovine, guinea pig, baboon and human species was separately incubated or perfused through a glass cannula with hydrocortisone, estrone, or testosterone. Chromatographic analysis showed that each of the hormones undergoes alterations in chemical structure. The trend is towards the formation of biologically inactive forms. As little as ten minutes incubation time was enough to show conversion products, which are thought to be enzymatic in nature.

Recent investigations have shown that estrone undergoes conversion during incubation in blood alone (1–3). These observations led to further studies to see if other steroids were similarly affected and if this was a species—specific or a general phenomenon.

An assessment of the potential importance of the vascular compartment as a steroid-metabolic "organ" was the prime interest, and for elucidation of this point the qualitative aspects of steroid transformation were of paramount importance. No effort was made to evaluate the exact chemical structure of the metabolites, since this represents an entirely different problem.

METHODS

Blood was drawn under aseptic conditions and, after defibrination with glass beads, was either incubated immediately or allowed to stand at 4° C for 24 hours to ascertain sterility and then incubated. Incubations consisted of (a) blood alone as the medium, (b) blood mixed

Received August 30, 1960.

[1] This investigation was supported by National Institutes of Health Grants Nos. A-1441, A-1078, and C-1609.

with an equal volume of Krebs-Ringer glucose phosphate buffer pH 7.4, or (c) blood mixed with White's solution. No cofactors or fumarate were used; the steroid was added as a finely powdered suspension or dissolved in a minute quantity of 95% ethyl alcohol. Incubations were carried out at 37.5° C for periods ranging from 10 minutes to 48 hours at 100 cpm oscillation. In addition, a mock perfusion of the blood (4) was used in the long-term experiments. The blood mixture was recycled through a glass cannula by a pump simulating the speed and pressure of aortic blood flow of the animal from which the blood was drawn. Control samples consisted of aliquots of the same blood used for the experiment but without added steroid.

The reactions were stopped with acetone and the control and experimental blood mixtures were extracted with chloroform and ethyl acetate and chromatographed on paper according to the methods of Zaffaroni (5) and Axelrod (6, 7). The colorless compounds were visualized by using the following spot test color reagents: 1. Blue tetrazolium (5) to detect the α-ketolic or dihydroxyacetone side chains of the corticosteroids. 2. Modified Zimmermann reagent (7) to demonstrate the presence of a ketone at carbon 17. 3. Dinitrophenyl-hydrazine (7) to detect α-β unsaturated 3-ketones or carbon 3-ketones. 4. Ferricyanide-ferrichloride mixture (8) to demonstrate phenolic groups. 5. Fuming sulfuric acid (15% free SO_3^-) (6) to detect, non-specifically, various steroid metabolites. 6. Antimony pentachloride reagent (6) to differentiate among some of the steroids.

RESULTS

The overall results of these experiments are shown in Tables 1–3. Although, as stated before, experiments were carried out from 10 minutes to 48 hours; the results herein shown were all present after 1 hr. of incubation. Although all spots visualized with the aid of the above tests are assumed to be results of the conversion of the steroid hormones, it should be noted that they need not necessarily be such. One possibility exists at least, that the added steroids displaced other components in the blood by competitive adsorption and that these latter non-steroidal compounds react with the functional group tests.

Hydrocortisone: Table 1 shows the results obtained after chromatography of the extracts of blood from 5 species incubated with hydrocortisone. Each zone shown on the table contains at least one α-ketolic steroid compound resulting from the conversion of hydrocortisone. The visual intensities of the B-T positive spots are graded as plus 1 to plus 5. The intensities from one chromatogram to another can be compared since aliquots of extract representing equal quantities of starting material (hydrocortisone) per volume of blood were chromatographed in identical fashion. The majority of metabolites are reduced in the α-β unsaturated 3-ketone position (as shown by the other spot tests) regardless of species. However, the total number of conversion products maintaining the α-ketolic side chain exceeds the theoretical maximum number of simple Δ^4-3-ketone reduction products (4 isomers) which could be obtained if only this portion of the molecule were attacked.

TABLE 1. α-KETOLIC METABOLITES OF HYDROCORTISONE[A] IN BLOOD[B]

Chromatogram zone (Decreased polarity ↓)	Mouse[C] ♀	Guinea pig ♂, ♀	Steer	Cow	Baboon ♂	Human ♂	Human ♀
1	3+	2+	1+	1+	1+	1+	1+
2	2+	3+	1+		1+	1+	1+
3	1+	2+	3+	2+	±	1+	2+
4	2+		1+	1+		±	1+
5	2+	3+	1+	1+	±	1+	1+
6	2+	1+	3+	1+	2+	2+	1+
7	2+	4+	2+	1+	1+	2+	2+

A = Chromatographed in the solvent systems toluene-propylene glycol for 7 days; and benzene-formamide 5 hrs.
B = Initial hormone concentration—6 mg./100 ml. blood.
C = C3H strain mice with no tumors.

TABLE 2. PHENOLIC METABOLITES OF ESTRONE[A] IN BLOOD[B]

Chromatogram zone	Mouse[C] ♀	Guinea pig ♀	Cow[D]	Human[E]
Decreased polarity ↓ 1	+	+		
2		+	+	+
3	+		+	
4		+	+	+

A = Chromatographed in the solvent systems o-dichoro-benzene-formamide and methylcyclohexane-propylene glycol.
B = Initial hormone concentration—2 mg./100 ml. blood.
C = C3H strain mice with no tumors.
D = Pregnant (4½–6 mos.)
E = Non-pregnant females, age: 29–36 years.

Estrone: The results of incubating estrone with separate blood mixtures of 4 species are depicted in Table 2. Each zone shown by a plus sign contains at least one phenolic compound not found in control blood. Area 2 in cow blood chromatograms has been shown to contain estradiol-17α (9). No evidence for the formation of estriol or its isomers has been obtained from these preparations.

Testosterone: Results of the incubation of testosterone in the blood of cows, steers, male and female guinea pigs and humans are shown in Table 3. No products indicating reduction of the α-β unsaturated 3-ketone of testosterone were found, nor was the oxidation product, 4-androsten-3, 17-dione, observed (10). Areas 1 and 2 includes compounds more polar than testosterone, indicating further oxygenation of the molecule.

DISCUSSION

To demonstrate the capacity of an organ to alter a hormone it is not requisite to identify the chemical structure of the product. In the present study it has been shown unequivocally that blood of various mammalian species can alter steroid hormones in a surprisingly short time, producing a multiplicity of conversion products. It is clear that eventually a complete description of the conversion products will be required; and the full significance of the conversion phenomena will not be known until the precise nature of the metabolites are established, and as stated above, that these compounds are truly conversion products.

The data available from our studies permit the following generalizations, however, with respect to the steroid conversion process:

TABLE 3. CONVERSION PRODUCTS OF TESTOSTERONE[A] IN BLOOD[B]

Chromatogram zone	Cow	Steer	Guinea pig ♂	Guinea pig ♀	♂
Decreased polarity ↓ 1	+	+	+	+	+
2		+			+
3				+	+

A = The plus sign represents conversion products in areas reacting to 2,4-dinitrophenylhydrazine and/or Zimmerman's reagent. Samples were chromatogramed in the solvent systems methylcyclohexane-propylene glycol and cyclohexene-formamide.
B = Initial hormone concentration—2 mg./100 ml. blood.

The reaction is rapid and probably enzymatic. Significant quantities of the hormones were converted in as little as 10 minutes incubation time. Since the steroid substrates were present in unphysiological concentrations, (2–6 mg./100 ml. blood), it is reasonable to assume that the enzymes involved are overloaded. Consequently, if these compounds were present in the blood at much smaller and more normal concentrations, the possibility of their existing therein in the forms used in these experiments would appear to be slight after a short time had elapsed, (given that no new hormones were elaborated by the endocrine glands and the metabolites were cleared by the kidney). Also, many of the conversion products which have "piled up" in the closed system would *in vivo* be converted to final products or excreted as such. It should also be noted that the trend in these metabolisms is for the hormone substrates to be converted to biologically less active forms (estrone to estradiol 17α, hydrocortisone to 'tetrahydro' forms, and testosterone to a more oxygenated structure).

Although in all species studied conversion products of the steroid substrates occurred, from the chromatographic and spot-test data, it is obvious that the conversion products and the relative amounts formed, are species-characteristic. Indeed, the data of Table 1 on hydrocortisone indicate even a difference in the manner in which steer blood metabolized hydrocortisone as compared to cow blood.

While the liver has the capacity to convert steroids in large quantity and with great rapidity, only a fraction of the circulating blood issuing from the heart passes through the liver at each pass. The amount, roughly calculated, is of the order of 10% per pass. On this basis the liver would need to have ten-fold the activity of the blood, at each pass, to be able to convert in a unit of time the same gross amount of hormone. The immediate secretions from the endocrine organs, in all cases, however, avoids the portal circulation, and the conversions which occur in the blood therefore, may be of equal if not greater significance to homeostasis than those which occur in other organs.

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LEUCINE AMINOPEPTIDASE IN THE GASTRIC MUCOSA OF THE ADRENALECTOMIZED RAT[1]

ABSTRACT

The effect of adrenalectomy on the gastric mucosa of the rat was studied by histochemical means. In the normal rat leucine aminopeptidase activity was relatively strong, particularly in the basal parts of the gastric glands. In the adrenalectomized animals activity was markedly decreased. It appears probable that leucine aminopeptidase is connected with the synthesis of pepsin in gastric glands, because it seems likely that this enzyme has a role in protein synthesis in tissues. In this instance the result agrees with observations made previously in a number of experimental and clinical studies.

The adrenal cortex modifies the secretory activity and histochemistry (1, 2) of the stomach. The cytology and structure of the gastric mucosa are altered by administration of cortical hormones or adrenalectomy (3). The importance of cortical hormones is indicated by the pathology which may arise under conditions of varying adrenocortical activity, e.g., after administration of ACTH to human patients latent peptic ulcers may become active (4).

The present study was carried out in order to investigate whether adrenalectomy in the rat influences histochemically demonstrable leucine aminopeptidase of the gastric mucosa. Nachlas, Crawford and Seligman (5) have shown a strong activity of this enzyme in the gastric mucosas of the rat, especially in the basal parts of gastric glands.

MATERIAL AND METHODS

Of twenty male adult albino rats, weighing about 230 gm., ten were adrenalectomized and ten used as controls. The operated rats and the control animals received drinking water and food *ad libitum*. The rats were killed by decapitation without anesthesia 10 days after the operation. The stomachs were removed immediately and frozen with dry ice. Sections were cut with the Pearse cold microtome at 8–30μ.

Leucine aminopeptidase activity was demonstrated by the method of Nachlas, Crawford and Seligman (5).

RESULTS

In sections from the animals of the control group strong leucine aminopeptidase activity was observed, especially in the basal parts of the gastric glands where the zymogenic cells are mainly located (Fig. 1). In the adrenalectomized rats activity was clearly decreased in the gastric mucosa. Occasionally, a slight purplish blue strain was discernible, which indicated a minimal activity of the enzyme (Fig. 2). This finding was made in all the operated animals.

DISCUSSION

Baker and Bridgman (3) showed that secretory activity is suppressed in the zymogenic cells of the stomach of the rat by adrenalectomy. These cytological observations agreed with the observation of Tuerkischer and Wertheimer (6) that gastric juice secreted in adrenalectomized animals under the influence of carbamylcholine chloride is low in pepsin, whereas administration of adrenocortical hormones increases the secretory activity of zymogenic cells. In patients receiving ACTH Spiro, Reifenstein and Gray (7) found an immediate and marked increase in the 24-hour excretion of uropepsin, a proteolytic enzyme originating in the stomach probably from pepsinogen. Total gastric resection abolished this effect. Patients who had adrenal insufficiency excreted no uropepsin in the urine and did not respond to ACTH by excreting any. Ingle, Sheppard, Oberle and Kuizenga (8) found deep ulcers in the pyloric portion of the stomach of rats that were treated with large doses of cortisol.

Received July 27, 1960.

[1] Supported by a grant from the Sigrid Jusélius Foundation, Helsinki.

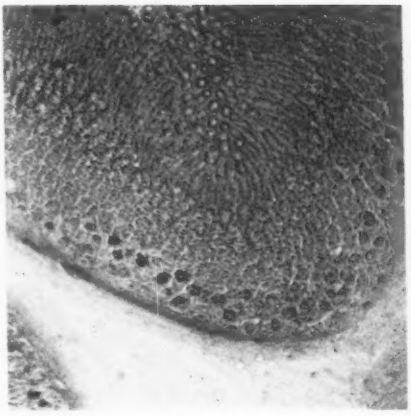

FIG. 1. Gastric mucosa of a control rat. There is a positive reaction for leucine aminopeptidase. Incubation time 30 minutes. ×200.

FIG. 2. Gastric mucosa of an adrenalectomized rat. Leucine aminopeptidase activity is depressed. Incubation time 30 minutes. ×200.

The physiological importance of leucine aminopeptidase is obscure. It is assumed that its activity in the tissues may be connected with production of proteins. In fact, Pearse and Tremblay (9) were able to demonstrate a presumptive direct relationship between the synthesis of the hormones of the parathyroid gland and the activity of leucine aminopeptidase. In our study, the reduced leucine aminopeptidase activity, may similarly be correlated with the lowered synthesis of the protein, pepsin.

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THE ENDOCRINE SOCIETY

The 1961 Annual Meeting

The Forty-Third Annual Meeting of the Endocrine Society will be held in the Hotel Biltmore, New York, N. Y., Thursday, Friday and Saturday, *June 22, 23 and 24, 1961.*

The Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 23, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Biltmore will hold 300 bedrooms for members until May 1, 1961, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 26 for the American Medical Association and other meetings, it is imperative that your reservations be made from June 26 on directly through the A.M.A. Housing Bureau. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1961.

Final program, membership card and advance registration forms will be sent on May 1, 1961 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to the Vice-President, Dr. Roy Hertz, National Cancer Institute, Bethesda, Maryland, not later than February 15, 1961. It is imperative that the abstracts be informative and complete with results and conclusions —not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee.

THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS, OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed two hundred words, or equivalent space,

exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.

3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words;
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)". Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)". The principal degree, e.g., M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.

ERRATUM

In the article entitled "Interpubic Ligament Regression in Relaxin-Treated Virgin and Primiparous, Post-Partum Mice" by E. H. Horn which appeared in *Endocrinology*, **67**: 668. 1960 the figure given in Table 2 for interpubic ligament length (mm.) of Group 7 should read 1.94 instead of 1.04.

SEVENTH INTERNATIONAL CONGRESS OF NEUROLOGY

(ROME, 10–15 SEPTEMBER 1961)

FIFTH INTERNATIONAL CONGRESS OF ELECTRO-ENCEPHALOGRAPHY AND CLINICAL NEUROPHYSIOLOGY

(ROME, 7–13 SEPTEMBER 1961)

The Seventh International Congress of Neurology will be held in Rome, September 10–15, 1961, under the auspices of the World Neurologic Federation and of the National Institute for Nervous Diseases and Blindness of Bethesda.

The Congress will have three categories of membership: *Active member*, open to members of the constituent national neurological societies; *Associate member*, open to qualified physicians and scientists having an interest in the Congress; *Adjunct member*, open to interested non-professionals and to families of members of the Congress. Registration fees are $15.00 (or equivalent in Italian lire) for Active members; $10.00 for Associate members, $5.00 for Adjunct members. Banquet and dance will be held in the Palace of "Tradizioni Popolari" Tuesday evening, September 12, 1961. Tickets for the banquet $8, dance $3, total $11, must be purchased at the time of registration and are not refundable.

Any information is available from the President of the Congress, Prof. Mario Gozzano or from the Secretary General, Dr. Giovanni Alemà, to the address of Viale Università 30, Rome. For the Symposium of Neurochemistry, information should be asked from Dr. Armand Lowenthal, Secretary for Neurochemistry, within the World Federation of Neurology, 59, rue Philippe Williot, Berchem-Anvers (Belgium). For the Symposium of Neurological Genetics information is obtainable from Prof. Luigi Gedda, Istituto, Gregorio Mendel, Piazza Galeno 5, Rome. For United States, designated from the United States Joint Committee on International Neurological Affairs as National Delegate is Dr. James L. O'Leary, M.D., Washington University School of Medicine, 600 S. Kingshighway, St. Louis 10, Mo., who will receive issue forms, registration applications and fees, and to receive and transmit communications to the Secretary General.

The Vth International Congress of Electroencephalography and Clinical Neurophysiology will be held from the 7th to the 13th September 1961.

Any information is obtainable from the President of the Congress, Prof. Mario Gozzano, or from the Secretary General, Dr. Raffaello Vizioli, Viale Università 30, Roma.

General Treasurer for both Congresses is Dr. Gian Franco Ricci, at the same address.